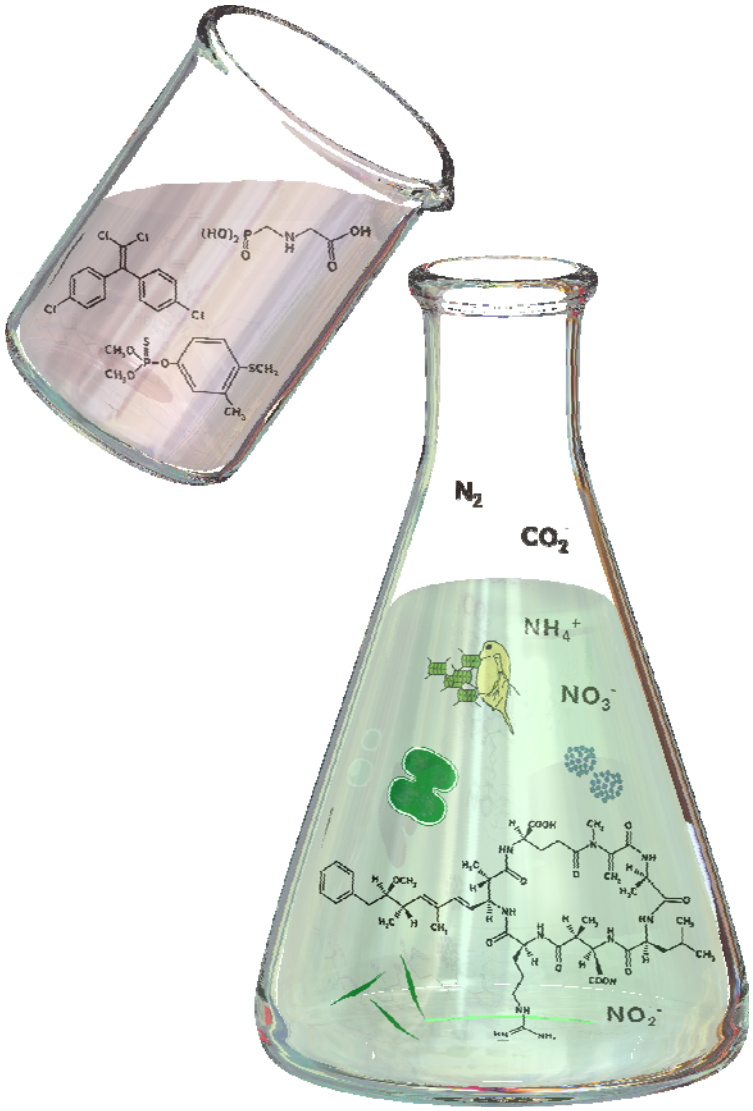
Hierarchical responses to organic contaminants in aquatic ecotoxicological bioassays: from microcystins to biodegradation



Katia Montenegro

Department of Ecology
Chemical Ecology and Ecotoxicology
Lund University
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Hierarchical responses to organic contaminants in aquatic ecotoxicological bioassays: from microcystins to biodegradation

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Dissertation
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A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have either already been published or are manuscripts at various stages (in press, submitted or in ms).

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This thesis is based on the following papers, which are referred to by their Roman numerals:

I. Bengtsson, G and Montenegro, K. Growth or microcystin production preserved in pesticide–exposed cyanobacteria?. (Manuscript)

II. Montenegro, K. and Bengtsson, G. Costs and benefits of toxin production in herbicide–stressed *Microcystis aeruginosa*. (Manuscript)

III. Bengtsson, G., Hansson, L-A. and Montenegro, K. 2004. Reduced grazing rates in *Daphnia pulex* caused by contaminants: implications for trophic cascades. Environmental Toxicology and Chemistry, 23, 2641-2648.

IV. Montenegro, K. and Bengtsson, G. Diversity – productivity relationships in pesticide–exposed Chlorophyta communities. (Manuscript)

V. Montenegro, K., Barmen, G. and Bengtsson, G. Contribution of groundwater residence time and biodegradation to persistence and effects of pesticides in aquifers. (Manuscript)

Introduction

The need to use ecological theories in ecotoxicology (putting the eco) is a continuous and old claim given the historical preference on the development and standardization of test systems, and on using traditional test results for risk assessment of chemicals, leaving behind to strengthen the scientific basis of the field (Cairns, 1988; Clements and Kiffney, 1994; Van Straalen and Løkke, 1997; Rohr et al., 2006).

The ecological consequences of environmental contamination are determined by a complex interplay of cause and effect at different hierarchical levels (Cairns et al., 1995). For example, contaminant effects occur at the individual level and the responses are manifested at the population level, so the dynamics of entities at one level of organization constitute the mechanisms that determine the dynamics at the next higher level of organization (Suter, 1995). Chemicals have unique effects at different levels of complexity and time-space scale, so the effects of contaminants identified will depend on what is measured, where it is measured, and how long it is measured (Cairns et al., 1995). Hence, without a better theoretical understanding of how effects at various levels of biotic and abiotic organization interact, we will stand little chance of making sense out of even the most standardized and harmonized data (Van Straalen and Løkke, 1997).

The traditional demographic parameters mortality, growth and fecundity rates are the most commonly used population endpoints in risk assessment (Suter, 1995). Among them, population growth rate is considered to provide the best summary statistics to measure the adverse impact of contaminants on the net population fitness given that it is the average result of changes in survival or fecundity of all individuals within the population (Sibly and Hone, 2002), and the most commonly used endpoint for algae (Lewis, 2003). In Paper I, the sensitivity of growth rate to detect effects of two organic contaminants was compared with the one of a novel endpoint based on cyanobacterial toxin production.

Stress, phenomena that all organisms regularly experience, can trigger changes in the dynamics of resource allocation by favouring one trait over others if metabolic costs are increased in response to stress. For example, there should be a trade-off between capacity to survive and growth rate or reproduction output (Calow, 1991). Trade-offs have received relatively little attention in ecotoxicological testing in spite of the impact that they may have on single traits such as survival, growth, and reproduction of species, which at the same time are the most commonly assessed endpoints (Benjamin and Klaine, 1995). In Paper II, I explore the combined impact of an herbicide and growth-limiting factors, such as light and phosphorus, in triggering a potential trade-off between growth and toxins production in cyanobacteria.

A prominent interaction in aquatic systems is between herbivores and producers (e.g., zooplankton–algae interaction) and has been the focus of many laboratory and field studies in basic and applied ecology, and one of the most clear cases in which contaminants can affect interspecific competition (species interaction) (Atchison et al., 1996; Relyea and Hoverman, 2006). The classical example is the phytoplankton bloom after competitive release (i.e., decreased grazing pressure) due to direct effects of insecticides on zooplankton (Hurlbert, 1975). In paper III I

quantify the direct (top-down trophic cascade) and indirect (bottom-up) impact of two contaminants on the interaction between green algae and *Daphnia*.

The characterization of species assemblages has a long history in environmental assessment and has helped to gain many insights about the impact of, for example, nutrient enrichment and xenobiotics, on the intermediate complex hierarchical level represented by communities (Schindler, 1987; Ford, 1989; Clements and Newman, 2002; Stevenson and Smol, 2003). The relationship between structural and functional properties of a community is a classical challenge in basic disturbance ecology that has been undertaken in ecotoxicology to understand the dynamics in responses to contaminants (Sheehan, 1984; Levine, 1989; Pratt and Cairns, 1996; Clements and Newman, 2002). In paper IV, the impact of two pesticides on the diversity–productivity relationship in Chlorophyta communities was clarified on a bivariate plane in a first attempt to my knowledge.

Contamination of groundwater by pesticides, their metabolites, and nitrate, is a result of the widespread use of agricultural chemicals around the world, especially in surficial (shallow) aquifers, and particularly beneath agricultural lands (Böhlke and Denver, 1995; Johnston et al., 1998; Johnson et al., 2003; Thayalakumaran et al., 2008). Microbial processes play important roles in nutrient cycling (e.g., nitrification and denitrification) and natural attenuation of contaminants (biodegradation) which are important determinants of ecosystem fertility and water quality (Alexander, 1994; Pell et al., 1998; Kemp and Dodds, 2002), especially in some environments as groundwater, where they may be the sole means of transformation (Alexander, 1994). In Paper V I attempted to estimate the contribution of residence time of groundwater to the persistence and effects of organic contaminants in aquifers along sites with a gradient of contamination in the selected area of León-Chinandega (Nicaragua).

I used the approach of calculating replicated point estimates of percentage inhibition (or stimulation) concentration to estimate the nature and magnitude of the effects of contaminants (Cooney, 1995), due to practical limitations and to the complexity in experimental designs that prevented me to develop traditional toxicity-testing that usually includes the calculation of EC50's and associated parameters for risk assessment. I focused mostly on supporting ecological epidemiology by providing mechanistic explanations that could be used to interpret the occurrence and magnitude of effects and prove causation at higher scales (Suter, 1995). I made an additional effort in comparing the magnitude of the effect of contaminants with the effects of natural stressors (biotic and abiotic) on the studied endpoints.

The general aim of the thesis was to gain new insights into ecotoxicological effects of organic contaminants on population and community interactions in aquatic environments. This was accomplished by the design and implementation of bioassays with aquatic organisms at different trophic levels to identify dose-effect relationships of selected pesticides and one metabolite. The bioassays used novel ecotoxicological endpoints or approaches at different hierarchical levels to elucidate the effects of exposure to contaminants and part of them were aimed at standardizing cost-effective (cheap and sensitive) methodology for routine chronic toxicity protocols. The experimental projects were developed following a sandwich model in a way that some of the bioassays were performed in Sweden to take advantage of the technology and to acquire knowledge, and others in Nicaragua to share experience with fellows at

CIRA-UNAN in an attempt to hybridize and exchange experience and technology to overcome limitations that permitted me to perform ecotoxicology in both places. The practical spin-offs of the thesis are *i*) to contribute to the advancement of Nicaraguan water quality guidelines for pesticides, and *ii*) to enforce legal restriction on import and use of internationally forbidden pesticides in Nicaragua.

The environmental sequels after the insecticide DDT, "the magic dust", and the modern success of "the once-in-a-century herbicide" glyphosate have inspired my almost constant effort in comparing the effects of the two compounds that have different physical chemical properties, and modes, and sites of action in the bioassays. The organic contaminants of interest in this thesis were in order of importance: the herbicide glyphosate, the insecticide metabolite pp'-DDE (the main metabolite of DDT), and the organophosphate insecticide fenthion. All are commonly found or widely used in my study area in Nicaragua (the León-Chinandega ecosystem).

Population ecotoxicology with the cyanobacteria *Microcystis aeruginosa* (Paper I)

Microcystins are the predominant natural toxins in freshwater lakes worldwide, produced by some cyanobacterial genus (e.g., *Microcystis*), and are considered as health hazards for wildlife and human populations (Carmichael, 1992; Chorus and Bartram, 1999). The absence of data on effects of pesticides or their metabolites on microcystin production motivated me to introduce it as a novel physiological endpoint for toxicity testing. I used the bioassay to test whether *i*) growth and toxin production of *M. aeruginosa* NIVA–CYA 228/1 were negatively related to the exposure concentrations of the contaminants (an insecticide metabolite and a herbicide) due to their expected inhibitory action on growth and the positive correlation between growth and toxin production, or *ii*) growth was more impaired than toxin production.

Glyphosate when dosed at the lowest concentration (Gly I=5 mg/L) reduced growth and MC–RR production (the main contributor to the MCs pool in the controls) in similar percentages, while increased the production of the most toxic microcystin analog MC–LR. When dosed at the highest concentration (Gly II=50 mg/L), it reduced growth and MC–LR production to the same extent, and at least extent MC–RR production. Additionally, when glyphosate was dosed at Gly I, it also affected the intracellular microcystin relative abundances by favouring the accumulation of MC–LR (from 15 to 50 %) over the accumulation of MC–RR (from 85 to 50 %). Light–limitation in the DDE bioassay produced a die-off in all cultures because they were performed in a fume hood. However, the treated cultures presented higher decay and MC–LR lost rates, and lower MC–RR production rates than the controls.

In summary, the accumulation of the most toxic analog MC–LR was favoured over the accumulation of the less toxic analog MC–RR under exposure to glyphosate at 5 mg L^{-1}, while MC–RR was the favoured one in light-limiting conditions and under exposure to pp'-DDE. This favouritism may depend on differential costs or affordability of production for each microcystin analog. The classical ecotoxicological endpoint, growth, was equally (MC–RR) or less sensitive (MC–LR) than microcystin production, but more robust and consistent in detecting the effects of glyphosate and pp'-DDE on *M. aeruginosa*. However, the poor temporal correlation of the

intracellular microcystin data, especially those of MC–RR, suggested us the possibility that both contaminants could have influenced microcystin export, either limiting or stimulating the releases to the surrounding media.

My contributions to the study of microcystin metabolism were:

i. To analyze the production of each analog independently, which in turn permitted me to notice the uncoupling between growth and microcystin production
ii. To provide evidences that organic contaminants may disrupt the coupling between growth and toxin production in cyanobacteria and favour the production of one analogue over the other
iii. To report that growth is a robust and consistent endpoint to detect effects of contaminants in *M. aeruginosa*

Trade-offs in population ecotoxicology: growth vs. toxin production in *M. aeruginosa* (Paper II)

The motivating results from the paper I inspired me to continue exploring the response to glyphosate in the relationship between growth and toxin production in an experiment designed to test for the existence and magnitude of a potential trade-off between the traits, combined with manipulation of different light regimes and phosphorus concentrations in order to compare their influence as driving-forces for the trade-off. However, this time, I used another strain of *M. aeruginosa*, the PCC 7806 (the wild type) that mainly produces the most toxic analogue MC–LR and to a minor extent its demethylated variant [D–Asp^3]MC–LR (less toxic), along with its mutant unable to produce microcystins, the PCC 7806 Δ*mcyB* (the mutant). Hence, I could calculate costs of microcystin production in metabolic terms (comparison of ATP cell quota in the mutant vs. wild type) and in ecological terms (ratio of growth rates in the mutant/wild type). Additionally, I measured the intracellular and extracellular microcystin pools during the three sampling occasions (0, 7 and 14 days).

The trade-off (negative functional interaction) was only detected when the cultures were incubated under photoperiods longer than 8 h of light (12:12 and 16:8), with differential patterns per microcystin analogue, time of incubation and exposure concentration. Growth was the definitively favoured trait during the first week of exposure, and less favoured over toxin production during the second week. The metabolic and ecological costs involved in microcystin production are higher during the first week of incubation and minimized afterwards. Glyphosate conferred additional ecological advantages to the mutants expressed in terms of tolerance, and especially when dosed at the highest tested concentration (40 mg/L). However, the herbicide seems also to confer benefits to the exposed wild type cultures in the second week of incubation by either increasing toxin production or inhibiting the extracellular releases to the medium. In general, the production of MC–LR seem to be favoured over the one of [D–Asp^3]MC–LR in the first seven days of incubation, and such favouritism minimized in the last week of incubation when both productions tended to be similar or even slightly higher for [D–Asp^3]MC–LR.

Glyphosate could not invert the nature of the interaction between both traits, neither in the phosphorus experiments (positive relationship) where cells were pre-P-starved, nor when the cultures were incubated under the shortest photoperiod of 8 h of light, where all controls and treatments had a negative interaction between traits. In the same experiments, glyphosate showed an impressive reduction of its herbicidal action on the cyanobacterial cells. The remarkable increased tolerance to glyphosate observed in the P experiments for both strains, more obvious however in the susceptible wild type cells, could be explained by the possibility that glyphosate was utilized as a phosphate source by cyanobacterial cells that were pre P–starved. This is in agreement with the findings that P–starved and glyphosate exposed *Anabaena variabilis* L. increased its extracellular alkaline phosphatase activity compared with a control and grew successfully (Ravi and Balakumar, 1998). The authors hypothesized that the high excretion of alkaline phosphatase into the medium cleaved the C–P bond in glyphosate and allowed *Anabaena* to utilize it as a phosphate source. However, this mechanism to detoxify and extract P at the same time is not general among cyanobacteria. The significantly higher ATP quotas in the wild type cells compared to the mutants in the first seven days of incubation in the P bioasssays, especially when the cells were re-inoculated in P-free-medium (Z8-), take me to hypothesize that the mutant probably has a poorer or less efficient ability to mobilize the stored phosphorus and transfer it to ATP, which is known to occur under phosphorus deficiency (Cembella et al., 1984). This may imply a potential role of microcystins into phosphorus storage dynamics.

The negative production rates calculated for [D–Asp^3]MC–LR, the rather constant relative contribution of the analogue to the intracellular microcystin pool, and the low extracellular concentrations detected during the first week of incubation in the light experiments might imply that the [D–Asp^3]MC–LR pool is not stable and that the compound is synthesized, used, and replenished constantly under the given culturing conditions. This turnover of the analogue seems to be triggered by glyphosate when the cultures were incubated under the 12:12 photoperiod (at 15 and 40 mg/L) and under the longest photoperiod (16:8, all treatments). Surprisingly the same phenomenon was also evident in controls and treatments of the cultures incubated under the shortest photoperiod (8:16), which additionally might imply that under light scarcity situations this turnover might be genetically induced in *M. aeruginosa*. The potential turnover of microcystins, only detectable for [D–Asp^3]MC–LR and not in MC–LR take me to hypothesize that there are differential costs of production per analogue. MC-LR is probably the more expensive to produce and hence conserved and seeming to be less labile than [D–Asp^3]MC–LR, given that was always the main contributor to the intracellular MCs pool, and only sacrificed under extreme situations like in the longest photoperiod (16:16) and under exposure to the highest glyphosate concentration. The different rates of metabolic turnover for the MC analogues is in agreement with varying turnover rates in allelopathic compounds, from high rates of the polyphenol phlototannin in tropical brown algae (Arnold and Targett, 2000) to low rates for terpenoids in terpene-accumulating families (e.g., *Mentha piperita* and *Pinus contorta*) (Gershenzon, 1994). This light-induced turnover of [D–Asp^3]MC–LR and the potential role of microcystins into phosphorus storage dynamics might imply multi-functional roles of the toxins, as suggested by Young et al. (2005) that by immunogold labelling localized microcystins preferentially associated with the thylakoids and polyphosphate bodies-periphery in my study strain.

Microcystins may also have an extracellular role as stress signalers, evidenced by the finding of the highest microcystin concentrations in the medium at the beginning of all the assays, especially in the pre P-starved cultures. Additional evidences for this possibility is the differential composition of the extracellular pool (50:50 or 60:40, MC–LR:[D–Asp^3]MC–LR) compared to the intracellular (70:30). The export of MCs has been usually been attributed to cell lysis (Paerl, 1988; Orr and Jones, 1998; Chorus and Bartram, 1999), but active excretion can't be ruled out given that a putative toxin exporter gene has been identified in the PCC 7806 strain (Pearson et al., 2004).

Growth seems to be a conservative trait in *M. aeruginosa*, which makes it the strongest endpoint to detect effects of contamination over microcystin production. However, microcystin production, and especially [D–Asp^3]MC–LR production was more responsive to glyphosate in time and at lower concentrations, that is, more sensitive, which is another desirable characteristic of ecotoxicological endpoints.

My contributions to the ecology of cyanobacteria were:

i. To test the existence and magnitude of a trade-off between growth and toxin production when the cultures were incubated under photoperiods typical from the tropics and during summer in temperate countries and after exposure to the herbicide glyphosate
ii. To quantify costs of microcystin production in metabolic (ATP) and in ecological terms (growth and tolerance to pollution)
iii. To provide evidences that *M. aeruginosa* has the potential capability to degrade glyphosate when cultures are pre-P starved
iv. To provide evidences of microcystin turnover ([D–Asp^3]MC–LR)
v. To provide evidences of the possible involvement of microcystins in three physiological roles: mobilization of stored phosphorus, stress signaler and light adaptation

Species interactions in ecotoxicology: *Daphnia*–algae (Paper III)

Daphnids are key species in freshwater ecosystems, e.g., as grazers on phytoplankton, and a favourite food item for many predators (Brett and R., 1996; Scheffer, 1998). Their ability to feed efficiently is at the same time their success and a major disadvantage because, in doing so, they are more exposed to contamination and thus more susceptible. The density-dependent grazing rate of *Daphnia pulex* pre-exposed to pp'-DDE (insecticide metabolite) and glyphosate (herbicide), via water or food (*Scenedesmus* spp.), was assayed in bioassays. The contaminant uptake and turnover pattern in *D. pulex* and *Scenedesmus* spp. was determined from parallel experiments with ^{14}C labeled compounds.

There was an inverse linear relationship between the net growth rate of *Scenedesmus spp*. and the density of *D. pulex*. In general, the slopes of the controls were steeper than for the treatments, suggesting that grazing became less efficient in *D. pulex* that were pre-exposed to the contaminants. The loss of grazing efficiency was, however, only significant for *D. pulex* pre-exposed to pp'-DDE via water (~30% compared to the control) and to glyphosate via *Scenedesmus* spp. (~40%). The

reduced grazing efficiencies were associated with the treatments that gave the highest body burden of pp'-DDE, and surprisingly, the lowest of glyphosate.

This decreased grazing efficiency after pre-exposure to glyphosate via food was not a direct effect of the contaminant on *D. pulex* as in the case of p,p'-DDE, but most likely an indirect effect probably mediated by the low concentration of glyphosate that *D. pulex* accumulated during the pre-exposure and metabolized during the experiments that in turn could have stimulated *Scenedesmus spp.* growth. Glyphosate, when dosed at very low concentrations similar to the one calculated from the ^{14}C-labelled parallel assays has been shown to stimulate chlorophyll-*a* synthesis in *S. quadricauda* (potential bottom-up effect), probably as a result of its nitrogen or phosphorus content, while at moderate concentrations it had no influence (Wong, 2000).

My contributions to ecotoxicology were:

i. To determine that grazing efficiency in *D. pulex* was more sensitive (15× for glyphosate and 3× for pp'-DDE) as an ecotoxicological endpoint than the 48h-LC50 for glyphosate and my estimated 48h-LC50 for pp'-DDE (13.5 µg/L)

ii. To provide evidences that indirect effects of contaminants may be at least as important as direct effects

Community ecotoxicology: diversity–productivity relationships in Chlorophyta (Paper IV)

I varied algal diversity (i.e., the number of species) and the concentration of the herbicide glyphosate and the organophosphate insecticide fenthion to test for effects of combinations of them on community productivity. Communities with different number of Chlorophyta species (2, 4, 8 and 16) but the same total initial biomass (~ 200 µg L^{-1} Chl) were exposed to three concentration levels of the pesticides (applied separately) during 7 days in laboratory bioassays. Genus representativeness and expected susceptibility /tolerance to glyphosate were considered in assembling communities in part of the batches, while in the rest, the communities were randomly composed.

Our general results showed that the relationship between diversity and productivity for the Chlorophyta communities was unimodal (concave), regardless of species composition, and composed by an ascending part that peaked at a diversity of 4 spp. and a descending part that usually ended at the highest diversity (16 spp.), but in some instances either saturated or saturated and continued decreasing. These results do not support any specific hypothesis for the relationship between diversity and productivity, but a hybrid of hypotheses including the diversity-productivity hypothesis, the redundant hypothesis, and the idiosyncratic hypothesis. This hump-shaping in the relationship probably happened due to competition for limiting resources in my initially dense communities that probably limit the environment carrying capacity to a number of available roles at 4 species to maximize productivity, but with potential to be between 4 and 7 spp. This is in agreement with observations of low primary producer diversity in many high productivity ecosystems around the

world, including algal blooms, salt and freshwater marshes, riparian forests in the tropics and temperate zones (Huston and McBride, 2004).

However, in two batches that had the same species composition, I observed idiosyncratic convex relationships with different amplitudes, surprisingly presenting their minimums at the observed maximums (4 spp.) of the hump-shaped relationship in the other batches after the replacement of a single species and hyperdominance of other species. These species-specific impacts on the diversity–productivity relationship were the hidden treatments in the bioassays, and highlighted the important roles of algae-bacteria associations (fenthion batch I and glyphosate batch II, *Cosmarium botrytis* case) and dominance (glyphosate batch II, *Chlorella fusca* case). The bacteria associated with the non-axenic culture of *C. botrytis* were capable of switching the concave relationship to a convex relationship, most likely by enhancing resource exploitation and competing with algae (including its host), due to the large portion of total uptake of inorganic nutrients that they account for (Kirchman, 1994), and hence reducing algal productivity (Loreau et al., 2001). However, the competitive effect of bacteria on algal productivity was slightly outweighed at the highest diversity (16 spp.), giving credit to the idea that biodiversity acts as a "biological insurance" against the disruptive effects of environmental fluctuations on ecosystem functioning (Loreau, 2000; Hector and Bagchi, 2007). The important role of primary producer-microbe interaction on the relationship between diversity and productivity has been recognized in algae-bacteria and arbuscular mycorrhizal (AMF) associations and usually explained by nutrient availability dynamics (Klironomos et al., 2000; Naeem et al., 2000; Van Der Heijden and Cornelissen, 2004).

Niche complementarities (Tilman et al., 1997; Loreau, 1998; Hector et al., 2004; Hille Ris Lambers et al., 2004), competition (Bond and Chase, 2002), and co-existence (redundancy, Loreau, 2000; Mouquet et al., 2002) may provide mechanistical explanations to the hybrid patterns of the diversity–productivity relationship, and explain the nature and strength of the operative species interactions. These general hypotheses and insights can be used to interpret the patterns graphically on a bivariate plane based on a verbal model inspired by a Bond and Chase's idea (2002) (Fig. 1): *i*) an ascending part may imply the absence of competition for resources (i.e., niche complementarities), *ii*) asymptotes may imply redundancy and mechanisms that promote co-existence, and *iii*) a descending part might imply complete competition for resources with steepness depending on the degree of competition.

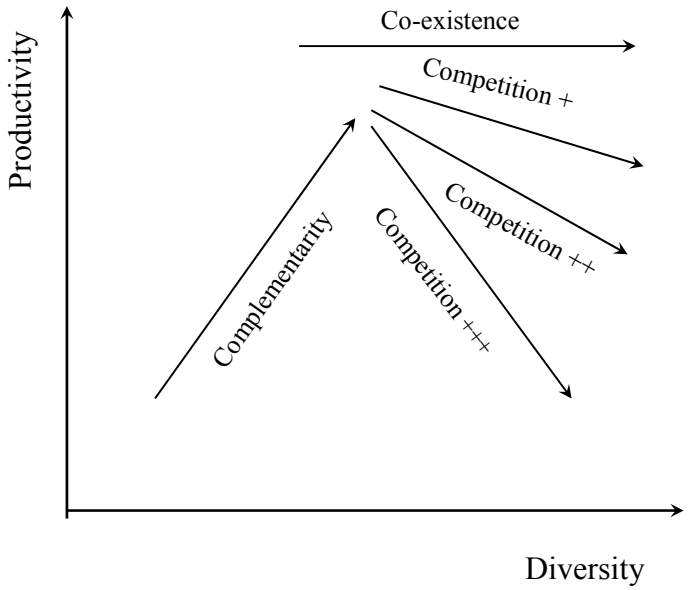

Figure 1. Graphical representation of the mechanisms that could shape and explain diversity–productivity relationships

Glyphosate did not change the trajectory of the relationship at the lowest and intermediate diversities, but at the highest diversities acting usually as a buffer for competition and otherwise as a provoker. The herbicide had an important impact on the magnitude of the effect of diversity on productivity sharpening the relationship probably by subsidizing it at almost all assayed concentrations, including its EEC (Expected Environmental Concentration) for aquatic exposure, and especially at the highest concentration (50 mg L^{-1}). This was partially expected because the herbicide may be used as a nutrient source and produce stimulatory effects in primary producers when dosed at low concentrations (Wong, 2000; Paper III; Cedergreen et al., 2007; Velini et al., 2008), but this does not apply to the highest assayed concentration. This was probably influenced by the high initial density of the cultures, which might have caused important biodilution of the herbicide (Paper III) even at the highest assayed concentration. *Pediastrum boryanum* and *Tetraedron minimum* were not found in one assemblage treated with the highest concentration, with no obvious impact on productivity. The results obtained in two attempts, however, brings out the possibility that glyphosate may have the ability to saturate productivity at lower levels of diversity (shifting the hump from 8–10 spp. to 4–5 spp.) and change the form of the relationship from asymptotic to unimodal, probably due to its potential to be utilized as a nutrients source.

Fenthion as expected had a lower impact on the effect of diversity on productivity than the one exerted by glyphosate because it is an insecticide, did not cause species extinctions, and also did not greatly change the pattern of the relationship. However, it tended to diminish productivity when dosed at concentrations above its EEC for aquatic exposure (100–1000 μg L^{-1}), especially in the most productive assemblages, and to increase it at intermediate levels of diversity. Fenthion is a hydrophobic compound and expected to affect algae only at high

concentrations (~1000 μg L^{-1}) (USEPA, 1998; Yeh and Chen, 2006), most likely due to its disorganization of chloroplasts (Yukimoto 1983). This suggests that the relationship is more responsive (sensitive) to glyphosate and fenthion than single species toxicity tests (No Observed Effect Concentration, NOEC = 11 mg/L and 200 μg/L, respectively).

Species composition, on the other hand, had an impact in the level of productivity reached in the assemblages hence affecting the amplitude of the relationship diversity–productivity, probably through inclusion or exclusion of productive or susceptible/tolerant species. In general, my results suggest that declining and augmenting diversity may result in local changes in productivity that may be enhanced or buffered by pesticides and easily detectable on a bivariate plane.

My contributions to ecotoxicology in this bioassay were:

i. To determine graphically the effect of diversity on productivity in the presence of pesticides
ii. To provide evidences that Chlorophyta can use glyphosate as a nutrient source
iii. To provide evidences that algae-bacteria interactions are stronger than pesticides in changing the trajectory of the relationship diversity–productivity at a local scale
iv. To provide evidences that dominance can buffer the trajectory of the relationship diversity–productivity

Processes that determine water quality in ecotoxicology: biodegradation and nitrogen cycling (Paper V)

The general objective of this study was to estimate the contribution of residence time of groundwater and *in situ* biodegradation potential to the persistence and effects of contaminants in aquifers of the selected area of León-Chinandega. To fulfill the objective we collected ground waters to provide natural inoculates for the biodegradation, nitrification and denitrification assays, and to be analyzed for water stable isotopes (Oxygen-18 and Deuterium) and chlorofluorocarbons to determine recharge sources and apparent ages.

The isotopic results identified three major sub-zones of recharge: the high altitude recharge area that received small contribution (20 %) from sources below 280 masl (the pristine deep well), the plains that received an equal contribution of recharge from high and low altitudes (50 %), and the low altitude discharge area that received the most substantial contribution (75–98 %) from sources below 280 masl.

The apparent age of ground waters is in average 23 years with a range from 16 to 29 years. The apparent CFC ages could not discriminate between the groundwater from the deep aquifer that was expected to be the oldest and the ground waters from the plains that were expected to be younger, probably because of the complexity of the system and the high degree of mixing of waters recharged at different elevations. The youngest ages derived from CFC-11 concentrations at all studied sites indicate potential degradation of the chlorofluorocarbon, especially in the discharge area and

most likely due to poor oxygen conditions. One site situated in the middle of irrigated fields presented the youngest water probably due to a higher turn over of the water.

Both the pristine deep aquifer and especially the shallow contaminated aquifer have degradation potential for DDE and glyphosate, and the most contaminated site may have strains adapted for faster degradation. This was expected since the microbial activity is expected to be higher in shallow aquifers compared to deep pristine aquifers, and that degradation occurs more readily at sites previously exposed to the substrate due to adaptation (Cavalier et al., 1991; Anderson, 1997).

The moderately contaminated sites tested had the highest potentials for nitrification and denitrification, and the most heavily contaminated the lowest for nitrification, most probably due to the presence of toxaphene. The three sites located at the discharge area presented higher denitrifying activity than the spring located in the plains which coincides with the lower *in situ* NO_3 concentrations in the former sites. Denitrification was less affected by contamination than nitrification.

The persistence of the *in situ* contaminants is not explained by the apparent age of water or the provenience. A combination of age tracers should be used to resolve the age distribution in the area due to the complexity of flow paths and mixing of waters.

My contributions with this study were:

i. To show that, in nature, microbial processes are responsive to contamination.

ii. To report that there is microbial indigenous potential for degradation of contaminants in all the aquifers assayed, especially in the most contaminated groundwater in the form of a higher degree of adaptation to degradation

iii. To confirm that nitrification and denitrification are sensitive endpoints to detect effects of contaminants, especially nitrification

iv. To report important degradation of CFC-11 in sub-oxic conditions in tropical aquifers

References

Alexander, M., 1994. Biodegradation and bioremediation. Academic Press Inc., London.

Anderson, T. A., 1997. Ecology and biogeochemistry of *in situ* groundwater bioremediation. In Jones (Eds.), Advances in microbial ecology, Plenum Press, New York.

Arnold, T. M., and Targett, N. M., 2000. Evidence for metabolic turnover of polyphenolics in tropical brown algae. J. Chem. Ecol. 26, 1393-1409.

Atchison, G. J., Sandheinrich, M. B., and Bryan, M. D., 1996. Chapter 11: Effects of stressors on interspecific interactions of aquatic animals. In Newman, M. C. and Jagoe, C. H. (Eds.), Ecotoxicology: a hierarchical treatment, CRC Press, Inc., Boca Ratón, pp. 319-338.

Benjamin, R. B., and Klaine, S. J., 1995. Fitness trade-offs of *Selenastrum capricornutum* strains selected for rapid growth on copper-spiked solidified nutrient medium. Environ. Toxicol. Chem. 14, 1789-1798.

Böhlke, J. K., and Denver, J. M., 1995. Combined use of groundwater dating, chemical, and isotopic analyses to resolve the history and fate of nitrate contamination in

two agricultural watershed, Atlantic coastal plain, Maryland. Water Resour. Res. 31, 2319-2339.
Bond, E. M., and Chase, J. M., 2002. Biodiversity and ecosystem functioning at local and regional spatial scales. Ecol. Lett. 5, 467-470.
Brett, M. T., and R., G. C., 1996. A meta-analysis of the freshwater trophic cascade. Proc. Natl. Acad. Sci. USA 93, 7723-7726.
Cairns, J. J., 1988. Putting the Eco in Ecotoxicology. Regul. Toxicol. Pharmacol. 8, 226-238.
Cairns, J. J., Niederlehner, B. R., and Smith, E. P., 1995. Ecosystem effects: functional end points. In Rand, G. M. (Eds.), Fundamentals of aquatic toxicology, Taylor & Francis, London, pp. 589-607.
Calow, P., 1991. Physiological costs of combating chemical toxicants: ecological implications. Comp. Biochem. Physiol. 100C, 3-6.
Carmichael, W. W., 1992. Cyanobacteria secondary metabolites- the cyanotoxins. *J. Appl. Bacteriol.* **72**, 445-459.
Cavalier, T. C., Lavy, T. L., and Mattice, J. D., 1991. Persistence of selected pesticides in ground-water samples. Ground water 29, 225-231.
Cedergreen, N., Streibig, J. C., Kudsk, P., and Mathiassen, S. K., 2007. The ocurrence of hormesis in plants and algae. Dose Response 5, 150-162.
Cembella, A. D., Antia, N. J., and Harrison, P. J., 1984. The utilization of inorganic and organic phosphorus compounds as nutrients by eukaryotic microalgae: a multidisciplinary perspective: Part 2. CRC Crit. Rev. Microbiol. 11, 13-81.
Chorus, I., and Bartram, J., 1999. Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. WHO, Suffolk.
Clements, W. H., and Kiffney, P. M., 1994. Assessing contaminant effects at higher levels of biological organization. Environ. Toxicol. Chem 13, 357-359.
Clements, W. H., and Newman, M. C., 2002. Community ecotoxicology. John Wiley & Sons Ltd., Chichester.
Cooney, J. D., 1995. Chapter 2: Freshwater tests. In Rand, G. M. (Eds.), Fundamentals of aquatic toxicology: effects, environmental fate and risk assessment, Taylor & Francis, Washington, pp. 71-99.
Ford, J., 1989. The effects of chemical stress on aquatic species composition and community structure. In Levin, S. A., Harwell, M. A., Kelly, J. R. and Kimball, K. D. (Eds.), Ecotoxicology: problems and approaches, Springer-Verlag, New York, pp. 99-144.
Gershenzon, J., 1994. Metabolic costs of terpenoid accumulation in higher plants. J. Chem. Ecol. 20, 1281-1328.
Hector, A., and Bagchi, R., 2007. Biodiversity and ecosystem multifunctionality. Nature 448, 188-191.
Hector, A., Loreau, M., Schmid, B., and Biodepth, 2004. Biodiversity manipulation experiments: studies replicated at multiple sites. In Loreau, M., Naeem, S. and Inchausti, P. (Eds.), Biodiversity and ecosystem functioning: synthesis and perspectives, Oxford University Press, pp. 36-46.
Hille Ris Lambers, J., Harpole, W. S., Tilman, D., Knops, J., and Reich, P.,2004. Mechanisms responsible for the positive diversity-productivity relationship in Minnesota grasslands. Ecol. Lett. 7, 661-668.
Hurlbert, S. H., 1975. Secondary effects of pesticides on aquatic ecosystems. Residue Rev. 57, 81-148.
Huston, M. A., and McBride, A. C., 2004. Evaluating the relative strengths of biotic versus abiotic controls on ecosystem processes. In Loreau, M., Naeem, S., Inchausti, P. (Eds.), Biodiversity and ecosystem functioning: synthesis and perspectives, Oxford University Press, pp. 47-60.
Johnson, A. C., White, C., Bhardwaj, C. L., and Nixon, A., 2003. The ability of indigenous micro-organisms to degrade isoproturon, atrazine and mecocrop within aerobic UK aquifer systems. Pest. Manag. Sci. 59, 1291-1302.

Johnston, C. T., Cook, P. G., Frape, S. K., Plummer, L. N., Busenberg, E., and Blackport, R. J., 1998. Ground water age and nitrate distribution within a glacial aquifer beneath a thick unsaturated zone. Ground water 36, 171-180.

Kemp, M. J., and Dodds, W. K., 2002. Comparisons of nitrification and denitrification in prairie and agriculturally influenced streams. Ecol. Applic. 12, 998-1009.

Kirchman, D. L., 1994. The uptake of inorganic nutrients by heterotrophic bacteria. Mic. Ecol. 28, 255-271.

Klironomos, J. N., McCune, J., Hart, M., and Neville, J., 2000. The influence of arbuscular mycorrhizae on the relationship between plant diversity and productivity. Ecol. Lett. 3, 137-141.

Levine, S. N., 1989. Theoretical and methodological reasons for variability in the responses of aquatic ecosystem processes to chemical stresses. In Levin, S. A., Harwell, M. A., Kelly, J. R. and Kimball, K. D. (Eds.), Ecotoxicology: problems and approaches, Springer-Varlag, New York, pp. 145-179.

Lewis, M. A., 2003. Algae and vascular plant tests. In Rand, G. M. (Eds.), Fundamentals of aquatic toxicology: effects, environmental fate and risk assessment, Taylor & Francis, Washington, pp. 135-169.

Loreau, M., 1998. Biodiversity and ecosystem functioning: a mechanistical model. Proc. Natl. Acad. Sc. U.S.A. 95, 5632-5636.

Loreau, M., 2000. Biodiversity and ecosystem function: recent theoretical advances. Oikos 91, 3-17.

Loreau, M., Naeem, S., Inchausti, P., Bengtsson, J., Grime, J. P., Hector, A., Hooper, D. U., Huston, M. A., Rafaelli, D., Schmid, B., functioning: current knowledge and future challenges. Science 294, 804-808.

Mouquet, N., Moore, J. L., and Loreau, M., 2002. Plant species richness and community productivity: why the mechanisms that promotes coexistence matters. Ecol. Lett. 5, 56-65.

Naeem, S., Hahn, D. R., and Schuurman, G., 2000. Producer-decomposer co-dependency influences biodiversity effect. Nature 403, 762-764.

Orr, P. T., and Jones, G. J., 1998. Relationship between microcystin production and cell division rates in nitrogen-limited *Microcystis aeruginosa* cultures. Limnol. Oceanogr. 43, 1604-1614.

Paerl, H. W., 1988. Growth and reproductive strategies of freshwater blue-green algae (cyanobacteria). In Sandgren, C. D. (Eds.), Growth and reproductive strategies of freshwater phytoplankton, Cambridge University Press, Cambridge, pp. 261-315.

Pearson, L. A., Hisbergues, M., Börner, T., Dittmann, E., and Neilan, B. A., 2004. Inactivation of an ABC transporter gene, *mcyH*, results in loss of microcystin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806. Appl. Environ. Microbiol. 70, 6370-6378.

Pell, M., Stenberg, B., and Torstensson, L., 1998. Potential denitrification and nitrification tests for evaluation of pesticide effects in soil. Ambio 27, 24-28.

Pratt, J. R., and Cairns, J. J., 1996. Chapter 12: Ecotoxicology and the redundancy problem; understanding effects on community structure and function. In Newman, M. C. and Jagoe, C. H. (Eds.), Ecotoxicology: a hierarchical treatment, CRC Press, Inc., Boca Ratón, pp. 347-370.

Ravi, V., and Balakumar, T., 1998. Biodegradation of the C-P bound in glyphosate by the cyanobacterium *Anabaena variabilis*. J. Sci. Ind. Res. (India) 57, 790-794.

Relyea, R., and Hoverman, J., 2006. Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. Ecol. Lett. 9, 1157-1171.

Rohr, J. R., Kerby, J. L., and Sih, A., 2006. Community ecology as a framework for predicting contaminant effects. Trends Ecol. Evol. (Amst.) 21, 606-613.

Scheffer, M., 1998. Trophic cascades. In Usher, M. B., De Angelis, D. L. and Manly, B. F. J. (Eds.), Ecology of shallow lakes, Chapman & Hall, London, pp. 122-146.

Schindler, D. W., 1987. Detecting ecosystem responses to anthropogenic stress. Can. J. Fish Aq. Sci. 44, 6-25.

Sheehan, P. J., 1984. Functional changes in the ecosystem. In Sheehan, P. J., Miller, D. R., Butler, G. C. and Bourdeau, P. (Eds.), Effects of pollutants at the ecosystem level, Scientific committee on problems of the environment (SCOPE), Norwich, pp. 101-145.

Sibly, R. M., and Hone, J., 2002. Population growth rate and its determinants: an overview. *Phil. Trans. R. Soc. Lond. B* **357**, 1153-1170.

Stevenson, R. J., and Smol, J. P., 2003. Use of algae in environmental assessments. In Wehr, J. D. a. S., R. G. (Eds.), Freshwater algae of North America, ecology and classification, Academic Press, San Diego, pp. 775-804.

Suter, G. W., 1995. Endpoints of interest at different levels of biological organization. In Cairns, J. J. and Niederlehner, B. R. (Eds.), Ecological toxicity testing: scale, complexity and relevance, pp. 35-48.

Thayalakumaran, T., Bristow, K. L., Charlesworth, P. B., and Fass, T., 2008. Geochemical conditions in groundwater systems: implications for the attenuation of agricultural nitrate. Agric. Water Manage. 95, 103-115.

Tilman, D., Knops, J., Wedin, D., Reich, P., Ritchie, M., and Siemann, E., 1997. The influence of functional diversity and composition on ecosystem processes. Science 277, 1300-1305.

USEPA, 1998. Reregistration eligibility decision Fenthion. EFED chapter. U.S. Environmental Protection Agency. Case 0290.

Van Der Heijden, M. G. A., and Cornelissen, J. H. C., 2004. The critical role of plant-microbe interactions on biodiversity and ecosystem functioning: arbuscular mycorrhizal associations as an example. In Loreau, M., Naeem, S., Inchausti, P. (Eds.), Biodiversity and ecosystem functioning: synthesis and perspectives, Oxford University Press, pp. 181-192.

Van Straalen, N. M., and Løkke, H., 1997. Ecological approaches in soil ecotoxicology. In Van Straalen, N. M. and Løkke, H. (Eds.), Ecotoxicological risk assessment of contaminants in soil, Chapman & Hall, London, pp. 3-18.

Velini, E. D., Alves, E., Godoy, M. C., Meschede, D. K., Souza, R. T., and Duke, S. O., 2008. Glyphosate applied at low doses can stimulate plant growth. Pest Manag. Sci. 64, 489-496.

Wong, P. K., 2000. Effects of 2,4-D, glyphosate and paraquat on growth, photosynthesis and chlorophyll-a synthesis of *Scedesmus quadricauda* Berb 614. Chemosphere 41, 177-182.

Yeh, H. J., and Chen, C. Y., 2006. Toxicity assessment of pesticides to *Pseudokirchneriella subcapitata* under air-tight test environment. J. Haz. Mat. A131, 6-12.

Young, F. M., Thomson, C., Metcalf, J. S., Lucocq, J. M., and Codd, G. A.,2005. Inmunogold localisation of microcystins in cryosectioned cells of *Microcystis*. J. Struct. Biol. 151, 208-214.

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Namasté!!!

Growth or microcystin production preserved in pesticide-exposed cyanobacteria?

Göran Bengtsson[a] and Katia Montenegro[a,b,]
[a]Department of Ecology, Lund University, Sölvegatan 37, SE-22362 Lund, Sweden
[b]CIRA-UNAN, Aptdo. Postal 4598, Managua, Nicaragua

Abstract

Mycrocystins are probably one of the most intensively studied natural toxins to date because of their impact on deterioration of water quality, hence the elucidation of mechanisms that explain the effects of stress factors on toxin production is urgent. Organic contaminants are known to reduce growth in cyanobacteria, but their effects on microcystin production are unknown, which motivated us to introduce toxins production as a novel physiological endpoint for toxicity testing and to compare its sensitivity with growth. We compared rates of growth and toxin production in batch cultures of *Microcystis aeruginosa* NIVA–CYA 228/1 under uncontaminated conditions (control) with those in which cultures were exposed to low and high (5 and 50 mg L^{-1}) concentrations of the herbicide glyphosate, and of the insecticide metabolite pp'-DDE (25 and 100 μg L^{-1}, respectively). The lowest concentration of glyphosate inhibited growth (30 %) and MC–RR production (25 %) in *M. aeruginosa*, while increased MC–LR production (50 %). At the highest glyphosate concentration tested, the herbicide completely inhibited all rates (130–165 %). pp'-DDE, on the other hand, increased the decaying rates of the cultures (50 %), decreased MC–RR production (60 %), and increased MC–LR loss (90 %). Glyphosate at the lowest assayed concentration also affected the intracellular microcystin relative abundances by favoring the accumulation of the most toxic analogue MC–LR over the least toxic MC–RR that is the known main contributor to the microcystins pool in the tested strain. The poor temporal correlation of intracellular microcystins data, especially those of MC–RR, suggested us the possibility that both contaminants could have influenced microcystin export, either limiting or stimulating the releases to the surrounding media. The classical ecotoxicological endpoint, growth resulted to have equal (MC–RR) or less (MC–LR) sensitivity than toxins production, but to be more robust and consistent in detecting the effects of both contaminants.

1. Introduction

Population growth rate is considered to provide the best summary statistics for the impact of pollutants on populations (Sibly, 2002), and is the most commonly used toxicological endpoint for algae (Shubert, 1984; Lewis, 1995). As cheaper and less cumbersome than other surrogates for growth (e.g., cell counts), chlorophyll parameters are used as cost-effective measurement endpoints for growth (Shubert, 1984; Marwood, 2001). Other physiological (e.g., photosynthesis) and biochemical endpoints (e.g., activity of enzymes) are helpful as early warning indicators that respond before growth effects are recognized and as an aid to identify mechanisms of pollutant effects at the population and community levels (Lin et al., 2005; Forbes et al., 2006).

Standardized official toxicity tests batteries (EC, 1993; USEPA, 2002; OECD, 2006) include at least one primary producer, and usually freshwater green algae (e.g., *Pseudokirchneriella subcapitata* Printz and *Scenedesmus* spp.) are most frequently used, although other species (e.g., the diatom *Naviculla pelliculosa*, and the cyanobacteria *Anabaena flos-aquae*) are also suitable but rarely used (Shubert, 1984; Lewis, 1995). Since the sensitivity to chemicals varies widely from one strain to another, ecological risk assessment based on conventional algal tests should include more species than those most frequently used (Wängberg and Blanck, 1988; Lewis, 1995).

The cosmopolitan genus *Microcystis* is a common cyanobacteria in algal blooms, and the majority of isolates synthesize one or more variants of microcystins and other toxic peptides, although non-producing isolates occur as well (Orr and Jones, 1998; Chorus and Bartram, 1999). Microcystins (MCs) is a group of at least 70 identified variants of cyclic heptapeptides, including methylated and demethylated products, in which two L–amino acids are variable and give their name to the microcystin(Chorus and Bartram, 1999). For example, MC–LR, one of the most toxic and abundant of the MCs, contains leucine (L) and arginine (R). Microcystins are toxic to mammals, protozoa and plants by inhibiting two major phosphatases used for growth and maintenance of the cell structure (Chorus and Bartram, 1999). That is probably why they are considered as secondary metabolites for anti-grazing defense (Carmichael, 1992; Chorus and Bartram, 1999), but they may also play a physiological role within the cell (e.g., light harvesters, trans-membrane transporters or chelators of metals) (Utkilen and Gjølme, 1995; Orr and Jones, 1998).

The rate of toxin production is positively correlated to cell division and growth rate, implying that microcystins are constitutively expressed (Orr and Jones, 1998; Long et al., 2001) and hence influenced by growth–limiting factors (e.g., light, temperature, and nutrient concentration) (Lukač and Aegerter, 1993; Rapala et al., 1997; Orr and Jones, 1998; Oh et al., 2000). Some of these environmental factors can also induce changes in the relative composition of microcystin variants and concomitant toxicity of some species (e.g., *M. aeruginosa* and *Anabaena* spp.) (Rapala et al., 1997; Oh et al., 2000). In contrast to most microbial secondary metabolites, MCs are produced from early exponential phase to stationary phase and are exported from the cells under certain environmental conditions or growth states (e.g., during lysis) (Paerl, 1988; Chorus and Bartram, 1999).

Whereas some pesticides are known to reduce growth in cyanobacteria (Hutber et al., 1979; Ravi and Balakumar, 1998; Forlani et al., 2008), nothing, to our knowledge, has been reported about their effects on microcystin production, motivating us to introduce toxins production as a novel physiological endpoint for toxicity testing. Additionally, the elucidation of mechanisms that explain the effects of contaminants on toxin production is urgent given the potential importance of microcystins in trophic interactions and their health hazards for wildlife and human populations (Carmichael, 1992; Chorus and Bartram, 1999).

The objective of the study was to examine the effects on growth and toxin production of a *Microcystis aeruginosa* strain from exposure to the herbicide glyphosate and the insecticide metabolite pp'-DDE. The objective was combined with an effort to design a batch culture bioassay for cyanobacterial species, in which the sensitivity of growth and toxin production as ecotoxicological endpoints was compared. Glyphosate is known to affect a variety of crucial processes in primary producers (e.g., protein synthesis, photosynthesis) which are first expressed as growth inhibition, followed by chlorosis and eventually death (Smith and Oehme, 1992). Although not primarily designed to act upon primary producers, insecticides and their metabolites (e.g., DDTs) may inhibit algal metabolism (e.g., photosynthesis) when associates with cell lipids and membranes, and subsequently reduce growth (Powers et al., 1979). We used the bioassay to test whether *i*) growth and toxin production of *M. aeruginosa* were both negatively related to the exposure concentrations of the contaminants due to their expected inhibitory action on growth and the positive correlation between growth and toxin production, or *ii*) growth was more impaired than toxin production because substantial amounts of energy are diverted from cell growth or division to maintenance processes in algae under stressed conditions (Gons and Mur, 1980). We also examined the effect of the contaminants on the relative abundances of two microcystins.

2. Materials and methods

2.1 Test species

The *Microcystis aeruginosa* (Kützing) Lemmermann strain NIVA–CYA 228/1 was donated by the Norwegian Institute for Water Research (http://www.niva.no/), and originally isolated from the eutrophic Lake Akersvatnet, Norway (NIVA, 1990). The strain produces mainly the microcystin variant [Dha^7]MC–RR or [D–Asp^3]MC–RR (demethylated variants of MC–RR without a methyl group in dehydroalanine, which is amino acid number 7, or aspartic acid, amino acid number 3, see next paragraph), and also some [Dha^7]MC–LR or MC–LR, and MC–YR (Mikalsen et al., 2003; Lyck, 2004). Batch cultures were maintained for three months in a ventilated hood under continuous white fluorescent light and intermittent aeration at ~ 20 ºC in Z8 medium, in which $NiSO_4(NH_4)_2SO_4{\cdot}6H_2O$ and $Al_2(SO_4)_3K_2SO_4{\cdot}24H_2O$ were replaced with equivalent molar amounts of $Ni(NO_3)_2{\cdot}6H_2O$ and $AlK(SO_4)_2{\cdot}6H_2O$, respectively (NIVA, 1990). Algal growth was calculated from measurements of the extracted chlorophyll *a* (Chl *a*) (Jespersen and Christoffersen, 1987) in an ultraviolet-visible spectrophotometer Ultrospec 3000 (Pharmacia Biotech, Cambridge, England).

Microcystins have a common cyclic structure usually containing three D–amino acids (alanine, *erythro*–β–methylaspartic acid and glutamic acid), two variable

L–amino acids and two unusual residues, *N*–methyldehydroalanine (Mdha), and the unique C_{20} amino acid residue (Adda), 3–amino–9–methoxy–2,6,8–trimethyl–10–phenyldeca–4*E*,6*E*–dienoic acid (Chorus and Bartram, 1999). The Adda moiety is responsible for MCs' toxicity and used for their identification and quantification (Orr and Jones, 1998; Chorus and Bartram, 1999). MCs vary in polarity with their substituted L–amino acids (Ward and Codd, 1999). For example, MC–LR is considered hydrophobic compared to the more polar MC–RR but hydrophilic compared to MC–LW (leucine–tryptophan) and –LF (leucine–phenylalanine). This may be important, since *in vivo* toxicity and hydrophobicity of microcystins is positively correlated (Ward and Codd, 1999). MC–LR and –RR standards were donated by Linda A. Lawton, School of Life Sciences, The Robert Gordon University, Aberdeen, UK. Additional MC–LR was purchased from Calbiochem (La Jolla, CA, USA). The toxins were dissolved in HPLC (High Performance Liquid Cromatography) grade methanol and ethanol (Fisher Scientific, Leics, UK), respectively. Aliquots of the stocks were diluted in methanol with 0.1% (v/v) trifluoroacetic acid (TFA, Sigma Chemical, St. Louis, MO, USA) to prepare working standard solutions for the HPLC analyses.

2.2 Test chemicals

Glyphosate (IUPAC name: N-[phosphonomethyl]glycine) is a broad-spectrum, nonselective systemic herbicide, which acts by effective inhibition of the 5-enolpyruvylshikimic acid-3-phosphate (EPSP) synthase, a mid-pathway enzyme of the aromatic amino acid biosynthesis, which links primary and secondary metabolism in prokaryotes, lower eukaryotes, and higher plants (Verschueren, 1983; Smith and Oehme, 1992). It is widely used in the world due to its efficacy and low potential for bioaccumulation or biomagnifications (Solomon and Thompson, 2003). Glyphosate (95% purity) was purchased from Sigma Chemical (St. Louis, MO, USA), and the stock solution (~ 5000 mg L^{-1}) was prepared in deionized water.

pp'-DDE (IUPAC name: 1,1-dichloro-2,2-bis[4-chlorophenyl]ethylene) is an impurity and the main dehydrochlorination metabolite of DDT (dichloro-diphenyl-trichloroethane) (Verschueren, 1983). In spite of its structural similarity to DDT, it has little insecticidal activity; however it affects other biochemical and biophysical processes in a similar way as DDT (Ferreira et al., 1997). DDT and metabolites are still found in environmental samples at higher levels than chronic toxicity thresholds (Ferreira et al., 1997). pp'-DDE (99% purity) was purchased from Sigma Chemical (St. Louis, MO, USA), and its stock solution (~ 400 mg L^{-1}) was prepared in acetone.

2.3 Microcystin extraction and analysis

Intracellular MCs were extracted as described by (Lawton et al., 2003) with some minor modifications. Depending on the thickness of the algal suspensions, 50 to 400 mL were filtered through 47 mm Ø Whatman GF/C (Maidstone, UK) glass microfibre filters. They were folded and placed into test tubes to be stored frozen at -20°C for at least 24 h. Thawed filters were extracted for 1 h with 4 mL of 100% methanol (HPLC grade) after vortexing for 30 sec at room temperature in darkness. The extract was transferred to another test tube, and the filter was squeezed to remove excess methanol, which was added to the extract. The extraction procedure was repeated twice. The three pooled extracts were concentrated to dryness in a vacuum centrifuge (SC110A SpeedVac®Plus, Savant Instruments Inc., Ohio, USA) at a medium drying rate (~ 40 ºC). The walls of the test tube were washed with methanol,

and the solution transferred to Eppendorf vials and centrifuged (Labofuge 200, Sepatech GmbH, Heraeus, Germany) for 15 min at 3000 rpm. The supernatant was transferred to new Eppendorf vials and re-concentrated in the vacuum centrifuge until the sample was dry. The residue was resuspended in 150 µL of methanol, the extracts centrifuged, and the supernatant transferred to HPLC vials.

The separation and quantification of the MC–RR and –LR microcystins was made by reversed phase in a Hewlett Packard HP Series 1050 HPLC (Hewlett Packard GmbH, Waldbronn, Germany) with 15 (pp'-DDE) or 35 min (glyphosate) sample runs and 10 min for post-column equilibration between the samples. The HPLC was equipped with a variable wavelength UV-VIS detector with 6.5 nm bandwidth that was set at 238 nm. The separation was carried out at room temperature (~ 20 ºC) on a 150×3 mm C18 Luna 5 µm (2) (Phenomenex Inc., CA, USA) column protected by a Phenomenex C18, 4×2 mm guard column. For the glyphosate experiments, the separation was achieved using a linear gradient elution of TFA-acidified water (60–70%) and acetonitrile (ACN, 30–40%) at a flow of 0.5 ml min^{-1} (Table 1). The gradient elution was changed to isocratic for the pp'-DDE experiment, since bubble formation in the pumping system following the increased proportion of ACN was affecting the reproducibility of areas and retention times. The new mobile phase consisted of a mixture of water (65%, 0.05 % TFA) and acetonitrile (35%, 0.1 % TFA) at a constant flow of 0.3 ml min^{-1} for 7 minutes, followed by a linear flow gradient from 0.3 to 0.6 ml min^{-1} (7–9 min), and from 0.6 to 0.3 ml min^{-1} (12–15 min). The MC concentrations were quantified from a calibration curve of three to five standards of a mixture of MC–RR (25 to 100 ng μl^{-1}) and –LR (2.5 to 10 ng μl^{-1}). The injection volumes varied from 10 to 30 µl depending on the toxin concentration. The estimated detection limits (DL) for MC–LR and –RR were 0.4 and 4 pg μl^{-1} (µg L^{-1}), respectively, for an injection volume of 10 µl and a filtered sample volume of ~ 200 ml.

Microcystin data were calculated as intracellular microcystin concentration per volume of algal suspension (µg MC L^{-1}) and then transformed to biomass–normalized microcystin concentration (toxin concentration divided by Chl *a*, dimensionless).

Table 1. Linear gradient elution conditions used for HPLC analysis in the glyphosate experiment. The flow rate was constant at 0.5 ml min^{-1}.

Eluent (%)	Time (min)					
	0	15	18	20	23	35
Water (TFA 0.05 %)	70	65	60	65	70	70
Acetonitrile (TFA 0.1 %)	30	35	40	35	30	30

2.4 Experimental setup

We compared the growth and toxin production of *M. aeruginosa* under uncontaminated conditions (control) with those in which cultures were exposed to 5 (Gly I) and 50 (Gly II) mg L^{-1} of glyphosate, and 25 (DDE I) and 100 (DDE II) µg L^{-1} of pp'-DDE in a Z8 growth medium during twelve days of incubation. The exposure to glyphosate was made in a temperature (20 ± 1 ºC) and light (16:8 h light:dark cycle) controlled room illuminated by four 58 W fluorescent lamps. We were forced to leave that room for the pp'-DDE experiment, which was performed in a fume hood at a similar temperature but under a light regime of 12 h per day provided by two 58 W fluorescent lamps. Aliquots of Z8 medium spiked with glyphosate or pp'-DDE were prepared 3 or 12 h before the exposures by magnetic stirring. An inoculum of *M. aeruginosa* from the stock culture was added to the Z8 medium to obtain 400 mL of approximately 250 µg L^{-1} Chl *a* (algal biomass in eutrophic conditions, (Chorus and Bartram, 1999) in each of 95 one litre glass jars per contaminant (30 for each exposure level and 35 for the controls). The jars were loosely covered with aluminum foil to allow some gas exchange but prevent aerial contamination and placed in rows of five replicates, in sequences (e.g., Control, Gly 5, Gly 50). Five control replicates were used to measure the Chl *a* and toxin content at the beginning of the assays. The 35 controls for the pp'-DDE batch contained some acetone. The samples were aerated for 2 h by a diaphragm air pump, stirred by hand and given new positions in their row every day. Five replicates per treatment and control from three adjacent rows of jars were destructively sampled for Chl *a* and intra–cellular toxin measurements every second day up to twelve days. The average specific growth rates and toxin production rates (day^{-1}) for both MCs in controls and treatments were derived from the slopes of the regression lines in plots of ln Chl *a* and ln [MC] (intracellular concentration) versus time of incubation (Orr and Jones, 1998; ECB, 2004). The average percentage inhibition or stimulation of mean specific growth or toxin production rates at each test substance concentration ($I\mu_t$) was calculated as $I\mu_t = [(\mu_c - \mu_t)/\mu_c] \times 100$, where μ_c is the mean control specific rate and μ_t is the mean specific rate for the test concentration t (ECB, 2004).

2.5 Data analyses

Linear regressions and associated parameter estimates (slopes and correlation coefficients) were calculated using the data analysis software STATISTICA version 6.1 (Stat Soft Inc. 2003, www.statsoft.com) at a significance level of 5 % (p=0.05). A confidence interval (95% probability) was used to delimit data amenable to a statistical comparison from each set of five replicates. Univariate ANCOVAs were used to examine the effects of chemicals on ln–transformed data of the three dependent variables (Chl *a*, MC– RR, and MC– LR), with time of incubation as covariate. The analysis was made using SPSS for Windows 9.0 (Chicago, IL, USA) at a significance level of 5 % (p=0.05). Simple contrasts were conducted to determine the significance of the differences in the means between treatments and controls in each bioassay. The significance of the interaction between group and time was used to assess the homogeneity of slopes (i.e., to assess differences in growth or production rates) between relevant pairs of regression lines (treatments vs. control). Data normality and homogeneity of variances were evaluated with Shapiro-Wilk's and Levene's tests, respectively.

3. Results

3.1 Growth

The growth of *Microcystis* was significantly reduced by glyphosate and even ceased in the Gly II cultures, which had visible symptoms of chlorosis (cells yellowing) (Table 2). The Chl *a* content of the control cultures increased from 250 to 3000 μg L^{-1} and to 1700 μg L^{-1} in the Gly I cultures during the twelve days of incubation, but decreased until 50 μg L^{-1} in the Gly II cultures (data not shown). The average percentage inhibition was ~ 30 % at Gly I and ~ 160 % at Gly II. The growth rates were negative in the DDE assay, and the treatments were 40–60 % more negative than the controls, with the highest inhibition in the DDE I treatment (Table 3). The Chl *a* concentrations decreased to 40–60 μg L^{-1} during the twelve days of incubation (data not shown). The die-off in the DDE assay reflected most likely sub-optimal light conditions. Both assays had high and significant temporal linear correlations with chlorophyll content (r^2= 0.65–0.99) implying that the derived growth or decay rates were creditable.

3.2 Microcystins

Only two peaks were clearly separated, quantifiable, and coincided in retention times with the available standards of MC–RR and MC–LR. The Gly I treatment produced 50 % more MC–LR than the control and 25 % less MC–RR (Table 2). The volumetric concentrations of MC–LR and MC–RR in the controls varied from 3 to 70 μg L^{-1} and from 15 to 100 μg L^{-1}, respectively, and from 2 to 100 μg L^{-1} and from 15 to 100 μg L^{-1}, respectively, in the Gly I cultures (data not shown). Both microcystins had negative production rates in the Gly II treatment implying a decrease in their concentrations by 130 % (MC–RR) and 160 % (MC–LR), respectively, compared with the control. The volumetric concentrations of MC–LR and MC–RR varied from 3 μg L^{-1} to < DL, and from 15 to 4 μg L^{-1}, respectively (data not shown). The MC–RR production rate in the DDE assay was ~ 3 times slower than in the glyphosate assay. The DDE II treatment reduced the concentration of both microcystins by 25–100 % compared with the control, and the MC–LR production ceased, even in the control (Table 3). The volumetric concentration of MC–RR varied from 110 to 400 μg L^{-1} in the assay, and the volumetric concentrations of MC–LR varied from 1 to 2 μg L^{-1} (data not shown). In general, the MC–LR intracellular concentrations in both assays presented higher and significant linear temporal correlations (r^2= 0.07–0.72) than the MC–RR concentrations (r^2= 0.0002–0.31), implying that the derived production rates were more creditable for the former than for the latter.

Table 2. Growth and toxin production rates in batch cultures of *Microcystis aeruginosa* NIVA–CYA 228/1 exposed during 12 days to two concentrations of glyphosate (Gly I=5 and Gly II=50 mg L^{-1}). The rates were derived from the slopes of the regression lines in plots of ln [Chl *a*] (growth) or ln [MC] (intracellular concentration, toxin production) versus time of incubation (days). One to five replicates within the 95 % confidence interval of the mean per treatment and day of exponential growth (or decay) were used.

Endpoint	Groups	Average specific growth or toxin production rate (day^{-1})	Linear correlation coefficient (r^2)	Significance of the difference in growth or toxin production rates (treatment vs. control)	Significance of the difference in group means (treatment vs. control)
Growth	Control	0.259	**0.99**		
	Gly I	0.188	**0.98**	p=0.00*	p=0.04*
	Gly II	-0.150	**0.66**	p=0.00*	p=0.06†
MC–RR production	Control	0.244	**0.30**		
	Gly I	0.186	0.16	p=0.67	p=0.90
	Gly II	-0.067	0.05	p=0.02*	p=0.39‡
MC–LR production	Control	0.340	**0.53**		
	Gly I	0.510	**0.72**	p=0.20	p=0.37
	Gly II	-0.216	**0.42**	p=0.00*	p=0.06‡

Bold: Correlation significant at p=0.05 †Levene's test p<0.05 ‡Shapiro-Wilk's test p<0.05

The glyphosate treatments had in general higher concentrations of MC–RR per biomass than their controls (Fig. 1A). More MC–LR was produced (or retained) per biomass in presence of 5 mg L^{-1} of glyphosate (Gly I) than in the control, and less or equal amounts in the presence of 50 mg L^{-1} of glyphosate (Gly II) (Fig. 2A). The toxins to biomass ratios were similar for control and treatments in the DDE bioassay, but the ratios were often lower for the control than for the treatments at the end of the assay (Figs. 1B, 2B). Considerably less MC–LR was retained per biomass (0.01–0.08) compared to MC–RR (» 1) in the DDE assay. The high ratios of MC–RR to Chl *a* indicate the decaying state of the cultures, since the maximum ratio for growing *M. aeruginosa* cultures is around 0.5 (Long et al., 2001; Lyck, 2004).

MC–LR made a minor contribution to the intracellular MCs pool (< 15 %) in the glyphosate assay and 1–2 % in the DDE bioassay in the control cultures during the exponential growth phase (< 12 days) (Fig. 3A), in agreement with observations by (Lyck, 2004) on exponentially growing *M. aeruginosa* cultures of the same strain (20 %). The MC–LR production was more stimulated than the MC–RR production by the Gly I treatment, and the contribution to the MCs pool went up to 50 %. The ratios of the MCs in the Gly II and the DDE treatments were invariant with those in the controls (Figs. 3A, 3B).

Table 3. Growth and toxin production rates in batch cultures of *Microcystis aeruginosa* NIVA–CYA 228/1 exposed during 12 days to two concentrations of pp'-DDE (DDE I=25 and DDE II=100 µg L^{-1}). The rates were derived from the slopes of the regression lines in plots of ln [Chl *a*] (growth) or ln [MC] (intracellular concentration, toxin production) versus time of incubation (days). One to five replicates within the 95 % confidence interval of the mean per treatment and day of exponential decay were used.

Endpoint	Groups	Average specific growth or toxin production rate (day^{-1})	Linear correlation coefficient (r^2)	Significance of the difference in growth or toxin production rates (treatment vs. control)	Significance of the difference in group means (treatment vs. control)
Growth	Control	-0.119	**0.65**		
	DDE I	-0.189	**0.82**	*p*=0.05*	*p*=0.13
	DDE II	-0.164	**0.74**	*p*=0.24	*p*=0.25
MC–RR production	Control	0.071	**0.31**		
	DDE I	0.053	**0.12**	*p*=0.72	*p*=0.31
	DDE II	0.002	0.0002	*p*=0.11	*p*=0.04*
MC–LR production	Control	-0.043	0.07		
	DDE I	-0.077	**0.54**	*p*=0.45	*p*=0.15
	DDE II	-0.088	**0.59**	*p*=0.33	*p*=0.13

Bold: Correlation significant at *p*=0.05

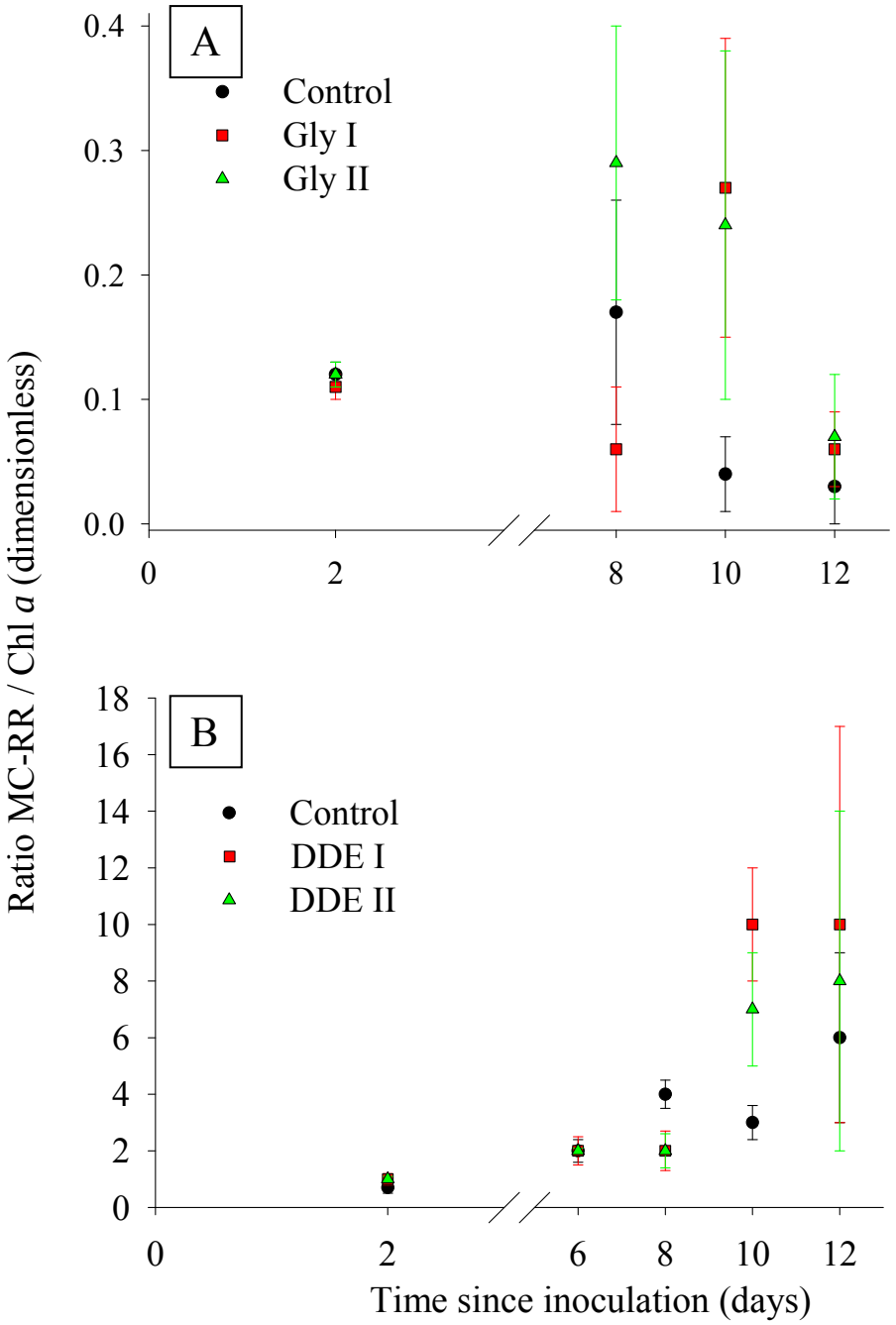

Figure. 1. Biomass normalized MC–RR content (toxin concentration to chlorophyll *a* ratio) in *Microcystis aeruginosa* NIVA–CYA 228/1 cultures vs. time since inoculation (days) in exposures to two levels of glyphosate (A) and pp'-DDE (B). Symbols represent the mean ratios of one to five replicates within the 95% confidence interval, and error bars represent the fractional standard deviation of the means.

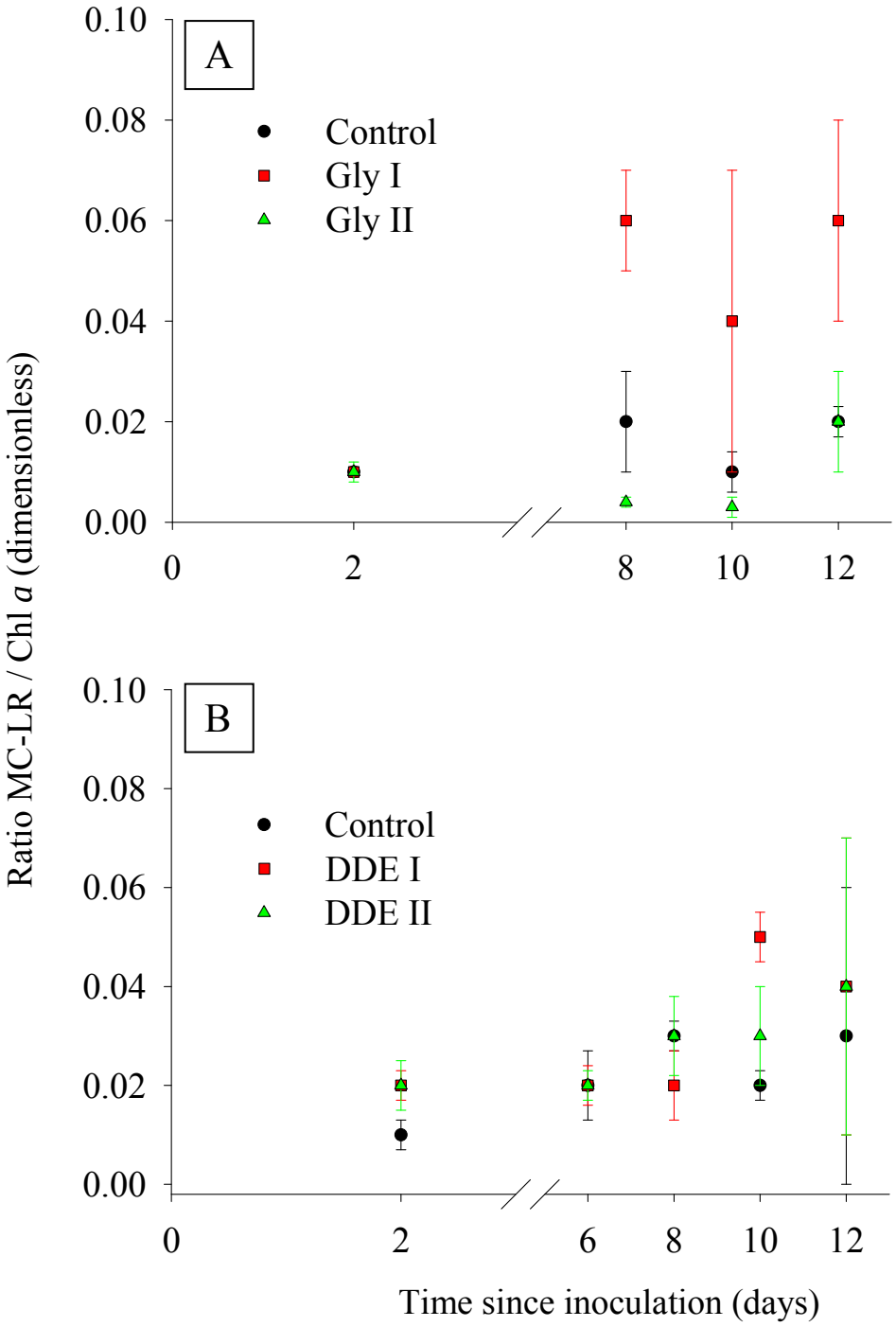

Figure 2. Biomass normalized MC–LR content (toxin concentration to chlorophyll *a* ratio) in *Microcystis aeruginosa* NIVA–CYA 228/1 cultures vs. time since inoculation (days) in exposures to two levels of glyphosate (A) and pp'-DDE (B). Symbols represent the mean ratios of one to five replicates within the 95% confidence interval, and error bars represent the fractional standard deviation of the means.

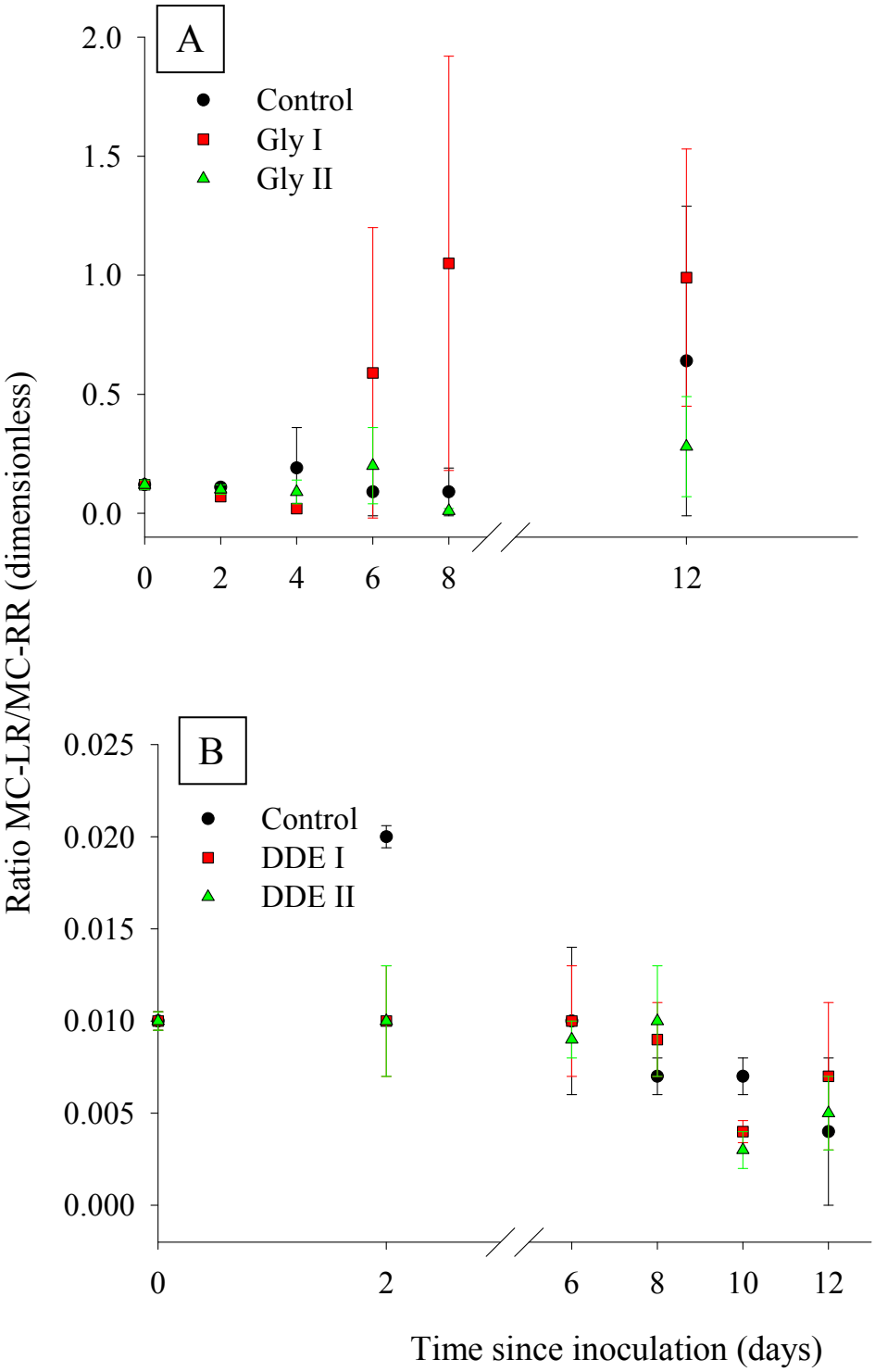

Figure 3. Ratio of MC–LR to MC–RR (dimensionless) in *Microcystis aeruginosa* NIVA–CYA 228/1 cultures vs. time since inoculation (days) in exposures to two levels of glyphosate (A) and pp'-DDE (B). Symbols represent the mean ratios of one to five replicates within the 95% confidence interval, and error bars represent the fractional standard deviation of the means.

4. Discussion and conclusions

Growth was the endpoint that consistently detected inhibitory effects and significant differences between controls and treatments in both bioassays, in addition to presenting robust data with high and significant temporal correlations. The mechanism for the growth reduction by glyphosate was probably inhibition of the chlorophyll synthesis, most likely mediated by the effects of the herbicide on its precursor ALA (5-aminolaevulinic acid) (Hoagland and Duke, 1982; Smith and Oehme, 1992; Ravi and Balakumar, 1998; Kannan et al., 1999). In general, Cyanophyta species are more sensitive to glyphosate than the most commonly used species (e.g., Chlorococcales) (Wängberg and Blanck, 1988) but have similar strain-to-strain variation. For example, growth of two *M. aeruginosa* strains was inhibited between 40 to 100 % by 30 and 60 mg L^{-1} of glyphosate (López-Rodas et al., 2007), and 40 mg L^{-1} added as Roundup® inhibited 100 % of the growth of *P. subcapitata* (Wängberg and Blanck, 1988). Our *M. aeruginosa* strain seemed to be quite sensitive, with an EC30 of 5 mg L^{-1}. Other cyanobacterial species, such as the filamentous N_2–fixers *Anabaena* spp. and *Nostoc* spp., have glyphosate EC50's for growth in the range of 2–11 mg L^{-1}, and EC100's for Roundup® in the range of 2–20 mg L^{-1} (Hutber et al., 1979; Wängberg and Blanck, 1988; Ravi and Balakumar, 1998), including the biofertilizer *Mastigocladus laminosus* (Kannan et al., 1999). The expression of tolerance to glyphosate by *M. aeruginosa* (growth inhibition at > 1 mM, 169 mg L^{-1}) by (Forlani et al., 2008) together to *Nostoc* and *Anabaena* strains was probably influenced by the extremely high initial densities and the long incubation time in the assays, which might have caused biodilution of the herbicide (Bengtsson et al., 2004). Another complication for the data interpretation is buffering (pH 7–8) of the growth medium or stock glyphosate solutions, as used by Forlani et al. (2008) and others reporting higher glyphosate EC50s (7.5–300×) for some of the same species (Powell et al., 1991; Cedergreen and Streibigand, 2005). Glyphosate is a zwitterion, with the mono–anion species predominating at low pH (< 6), and the more polar di–anion at high pH (6–10) (Cikalo et al., 1996). The di–anion is probably less toxic to algae than the mono–anion, explaining why also glyphosate formulations (e.g., Roundup® and Rodeo®) are more toxic to alga (5×) and bacteria (100×) at pH ≤ 4.6 than at pH ≥ 6.3 (Gardner et al., 1997; Amorós et al., 2007).

The poor performance of *M. aeruginosa* in the DDE assay reflects its sensitivity to small changes in environmental conditions, such as light reduction (Paerl, 1988; Lyck and Christoffersen, 2003). Cyanobacteria, and especially *M. aeruginosa* are known to have weaker darkness tolerance than eukaryotic algae (greens and diatoms) probably due to their inability to effectively use stored energy to maintain metabolic integrity (Furusato et al., 2004). They try to overcome the limitation by adjusting their vertical position in the water column by buoyancy alteration (Paerl, 1988). The additional die-off of the cultures in the presence of DDE may be explained by its inhibition of the photosynthesis and subsequently growth in algae (Powers et al., 1979).

No other studies are known to us on the effects of pesticides and their metabolites on microcystins production or yield, but glyphosate and its formulations increase the pool of free non–aromatic amino acids in carrot cells and spring wheat plants (Haderlie et al., 1977; Nilsson, 1977) and six out of ten of those free amino acids (especially glutamic and aspartic acids, and arginine) are precursors in the MC–

LR synthesis (Tillet et al., 2000). Similarly, overproduction of enzymes in yeast and fungi exposed to glyphosate increases the cellular pool of e.g., arginine and leucine (Delforge et al., 1975; Bode et al., 1984). and cellular proteolysis in the cyanobacterium *M. laminosus* exposed to Roundup® may cause the amino acid accumulation (Kannan et al., 1999).

The higher variability in the response of toxins production compared to growth, especially the one of MC–RR, could be explained by a distortion of our proxy (intracellular toxin content) due to an increased proportion of MCs released to the medium. This relative proportion of excreted microcystin to intracellular concentrations is also a debated subject; given than some few studies have reported important exports (20–75 %) (Orr and Jones, 1998; Wang et al., 2007), while the majority has reported insignificant looses (< 5 %) (Rapala et al., 1997; Long et al., 2001; Lyck and Christoffersen, 2003). The export of MCs has been usually attributed to cell lysis (Paerl, 1988; Orr and Jones, 1998; Chorus and Bartram, 1999), but active excretion can't be ruled out given that a putative toxin exporter gene has been identified in the PCC 7806 strain (Pearson et al., 2004). Furthermore, some environmental and chemical stressors are known to affect this export or release of microcystins. For example, low concentrations of nonylphenol (NP) were reported to diminish significantly the excretion of MC–LR (up to 45 % less than the control) with a concomitant increase of the intracellular content (75 % higher than the control) in actively growing cells of *M. aeruginosa* PCC 7820 (Wang et al., 2007). Curiously, the other strain tested, the PCC 562 did not export important amounts of the variant (< 1 %) under the same exposure conditions, and after reaching 2× higher densities than PCC 7820, suggesting a strain-specific strategy of microcystin releasing. These authors speculated that NP might have suppressed cell membrane receptors, and hence physiological activity (MC–LR active transport?) in the algal cells. On the other hand, Ross et al. (2006) reported that UV radiation, salinity, and the herbicide paraquat triggered microcystins release (40 to 90 %) compared to non–stressed cells (controls) in wild *M. aeruginosa* cells, with the herbicide eliciting the higher releases. The toxins release was explained by increased oxidative cell stress mediated by increased production of H_2O_2, which at the same time may trigger the initiation of programmed cell death or apoptosis. These reports give raise to suggest that there was a potentially differential export for each microcystin analog per bioassay, and that the contaminants might have either limited or stimulated microcystin releases compared to the controls, and hence produced the observed low temporal correlation of intracellular microcystins data.

Biochemical and physiological endpoints seem to be equally or more sensitive to glyphosate exposure than growth in cyanobacteria, in agreement with our observations on microcystins. For example, Ravi and Balakumar (1998) ranked *in vivo* nitrate reductase activity as the most sensitive endpoint, followed by Chl *a* and nitrogenase activity in assays with *A. variabilis* L. and *Nostoc* spp. L. Kannan et al. (1999) showed that nitrogen fixation in *M. laminosus* was 2.5× more affected by Roundup® than growth and photosynthesis. However, Hoagland (1985) noticed that inhibition of nitrate reductase is a secondary effect of glyphosate to growth inhibition. Unfortunately, these sensitive endpoints have two major practical limitations that severely compromise their cost-effectiveness: *i.* their results are not interpretable by their own but rather require substantial additional information, and *ii*. they require the use of sophisticated equipment (Lin et al., 2005; Forbes et al., 2006).

The tight coupling of cell division and microcystin production observed in nitrogen–limited batch cultures of MASH01-A19 strain of *M. aeruginosa* inspired Orr and Jones (1998) to generalize their findings to other cyanobacterial species as well, such that the microcystin production is indirectly affected by environmental factors (i.e., light, nutrients, and temperature) through their effects on cell division. Numerous exceptions are known. For example, Lyck (2004) found increased concentrations of intracellular microcystins (mainly [D–Asp^3]MC–RR) in light–limited batch cultures of our study strain, just as in the decaying cultures of the DDE assay. Severe P–limitation reduced net growth of chemostat cultures of the UTEX 2388 strain, but increased the intracellular content of MC–LR and MC–RR (Oh et al., 2000). Iron, when completely omitted from the medium in batch cultures of the PCC 7806 strain, decreased growth but increased intracellular MC–LR yield (Lukač and Aegerter, 1993). However, in iron-limited growth in continuous cultures of our study strain, Utkilen and Gjølme (1995) observed a decrease in the intracellular content of MC–RR, and in batch cultures MC–RR was more affected (25 %) than MC–LR (15 %). The contradictory observations call for approaches in which *i*. specific growth and microcystin production rates are compared instead of microcystin yield so that both traits are expressed on the same basis (day^{-1}) as suggested by (Orr and Jones, 1998), and *ii*. gross and net toxin production rates are calculated by measuring both the intra and extracellular pools per analog, in order to understand the relationship between growth and microcystin metabolism.

Increased intracellular ratios of MC–LR to MC–RR are known from other stress conditions in *M. aeruginosa* strains, such as severe phosphorus limitation (15 to 30 %, (Oh et al., 2000), and from iron limitation (40 to 45 %) (Utkilen and Gjølme, 1995), suggesting that xenobiotics, such as glyphosate, may influence the intracellular toxicity of *M. aeruginosa* at the same extent as nutrients. The increased intracellular toxicity has important implications for the zooplankton-cyanobacteria interaction since ingestion of cells containing microcystins (e.g., MC–LR) affect survival, growth and reproduction in grazers, while identical concentrations of the dissolved toxins do not (DeMott et al., 1991; Lürling and Van Der Grinten, 2003). DeMott et al. (1991), for example, reported that concentrations of dissolved MC–LR in the order of mg ml^{-1} were needed to cause mortality in daphnids compared to feeding them with intact cells that contained orders of magnitude lower concentrations.

The classical ecotoxicological endpoint, growth, was equally (MC–RR) or less (MC–LR) sensitive than microcystin production, but more robust and consistent in detecting the effects of glyphosate and pp'-DDE on *M. aeruginosa*. This study also provides evidence for the disruption of the expected coupling between population growth and microcystin production in *M. aeruginosa* by the herbicide glyphosate, and under light–limitation and exposure to the insecticide metabolite pp'-DDE. We also show that the accumulation of the most toxic analog MC–LR is favored over the accumulation of the less toxic analog MC–RR under exposure to glyphosate at 5 mg L^{-1}, while MC–RR was the favored one in light-limiting conditions and under exposure to pp'-DDE. This may depend on differential costs or affordability of production for each microcystin analog. Alternatively, MC–RR was probably preferentially exported in the glyphosate assay and MC–LR in the DDE assay, with major releases by the treatments; while the MC–LR export could have been

diminished by exposure to glyphosate. Future efforts are needed and should be devoted to mechanistically describe the per analog microcystin metabolism (i.e. production, export, and breakdown) by measuring the gross and net changes in intracellular and bulk (intra+extracellular) microcystins content.

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References

Amorós, I., Alonso, J. L., Romaguera, S., and Carrasco, J. M., 2007. Assessment of toxicity of a glyphosate-based formulation using bacterial systems in lake water. Chemosphere 67, 2221-2228.

Bengtsson, G., Hansson, L.-A., and Montenegro, K., 2004. Reduced grazing rates in *Daphnia pulex* caused by contaminants: implications for trophic cascades. Environ. Contam. Chem. 23, 2641-2648.

Bode, R., Melo, C., and Birnbaum, D., 1984. Enzymological basis for glyphosate action in *Candida maltosa*. Biochem. Physiol. Pflanzen 179, 775-783.

Carmichael, W. W., 1992. Cyanobacteria secondary metabolites- the cyanotoxins. J. Appl. Bacteriol. 72, 445-459.

Cedergreen, N., and Streibig, J. C., 2005. The toxicity of herbicides to non-target aquatic plants and algae: assessment of predictive factors and hazard. Pest Management Science 61, 1152-1160.

Chorus, I., and Bartram, J., 1999. Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. WHO, Suffolk.

Cikalo, M. G., Goodall, D. M., and Matthews, W., 1996. Analysis of glyphosate using capillary eletrophoresis with indirect detection. J. Chromatogr. A 745, 189-200.

Delforge, J., Messenguy, F., and Wiame, J.-M., 1975. The regulation of arginine biosynthesis in *Saccharomyces cerevisiae*. Eur. J. Biochem. 57, 231-239.

DeMott, W. R., Zhang, Q.-X., and Carmichael, W. W., 1991. Effects of toxic cyanobacteria and purified toxins on the survival and feeding of a copepod and three species of *Daphnia*. Limnol. Oceanogr. 36, 1346-1357.

EC, 1993. Biological test method: growth inhibition test using the freshwater alga *Selenastrum capricornutum*. Environment Canada, Ottawa. EPS 1/RM/25.

ECB, 2004. Methods for the determination of ecotoxicity: C.3. Algal inhibition test. EEC directive 92/69/EEC, Annex V, Part C. European Chemicals Bureau,

Ferreira, F. M. L., Madeira, V. M. C., and Moreno, A. J., 1997. Interactions of 2,2-bis(p-chlorophenyl)-1,1,dichloroethylene with mitochondrial oxidative phosphorylation. Biochem. Pharmacol. 53, 299-308.

Forbes, V. E., Palmqvist, A., and Bach, L., 2006. The use and misuse of biomarkers in ecotoxicology. Environ. Toxicol. Chem. 25, 272-280.

Forlani, G., Pavan, M., Gramek, M., Kafarski, P., and Lipok, J., 2008. Biochemical bases for a widespread tolerance of cyanobacteria to the phosphonate herbicide glyphosate. Plant Cell Physiol. 49, 443-456.

Furusato, E., Asaeda, T., and Manatunge, J., 2004. Tolerance for prolonged darkness of three phytoplankton species, *Microcystis aeruginosa* (Cyanophyceae), *Scenedesmus quadricauda* (Chlorophyceae), and *Melosira ambigua* (Bacillariophyceae). Hydrobiologia 527, 153-162.

Gardner, S. C., Grue, C. E., Graslley, J. M., Lenz, L. A., and Lindenauer, J.M., 1997. Single species algal (*Ankistrodesmus*) toxicity tests with Rodeo® and Garlon ® 3A. Bull. Environ. Contam. Toxicol. 59, 492-499.

Gons, J. H., and Mur, L. R., 1980. Energy requirements for growth and maintenance of *Scenedesmus protuberans* Fritsch in light-limited continuous cultures. Arch. Microbiol. 125, 9-17.

Haderlie, L. C., Widholm, J. M., and Slife, F. W., 1977. Effect of glyphosate on carrot and tobacco cells. Plant Physiol. 60, 40-43.

Hoagland, R. E., 1985. Influence of glyphosate on nitrate reductase activity in soybean (*Glycine max)*. Plant Cell Physiol. 26, 565- 570.

Hoagland, R. E., and Duke, S. O., 1982. Biochemical effects of glyphosate [*N*-(Phosphonomethyl)glycine]. In Moreland, D. E., St. John, J. B., Dana Hess, F. (Eds.), Biochemical responses induced by herbicides, ACS, Washington, D.C., pp. 175-205.

Hutber, G. N., Rogers, L. J., and Smith, A. J., 1979. Influence of pesticides on the growth of cyanobacteria. Z. Allg. Mikrobiol. 19, 397-402.

Jespersen, A. M., and Christoffersen, K., 1987. Measurements of chlorophyll a from phytoplankton using ethanol as extraction solvent. Arch. Hydrobiol. 109, 445-454.

Kannan, V., Srinivasan, S., Kathiravan, G., Raju, K., Subramanian, K., and Sivakumar, D., 1999. Influence of glyphosate on *Mastigocladus laminosus*. J. Environ. Biol. 20, 199-206.

Lawton, L. A., Robertson, P. K. J., Cornish, B. J. P. A., Marr, I. L., and Jaspars, M., 2003. Processes influencing surface interaction and photocatalytic destruction of microcystins on titanium dioxide photocatalysts. J. Catalysis 213, 109-113.

Lewis, M. A., 1995. Algae and vascular plant tests. In Rand, G. M. (Eds.), Fundamentals of aquatic toxicology: effects, environmental fate and risk assessment, Taylor & Francis, Washington, pp. 135-169.

Lin, J.-H., Kao, W.-C., Tsai, K.-P., and Chen, C.-Y., 2005. A novel algal toxicity testing technique for assessing the toxicity of both metallic and organic toxicants. Wat. Res. 39, 1869-1877.

Long, B. M., Jones, G. J., and Orr, P. T., 2001. Cellular microcystin content in N-limited *Microcystis aeruginosa* can be predicted from growth rate. Appl. Environ. Microbiol. 67, 278-283.

López-Rodas, V., Flores-Moya, A., Maneiro, E., Perdigones, N., Marva, F., García, M. E., and Costas, E., 2007. Resistance to glyphosate in the cyanobacterium *Microcystis aeruginosa* as result of pre-selective mutations. Evol. Ecol. 21, 535-547.

Lukač, M., and Aegerter, R., 1993. Influence of trace metals on growth and toxin production of *Microcystis aeruginosa*. Toxicon 31, 293-305.

Lürling, M., and Van Der Grinten, E., 2003. Life-history characteristics of *Daphnia* exposed to dissolved microcystin-LR and to the cyanobaterium *Microcystis*

aeruginosa with and without microcystins. Environ. Toxicol. Chem. 22, 1281-1287.

Lyck, S., 2004. Simultaneous changes in cell quotas of microcystin, chlorophyll *a*, protein, and carbohydrate during different growth phases of a batch culture experiment with *Microcystis aeruginosa*. J. Plankton Res. 26, 727-736.

Lyck, S., and Christoffersen, K., 2003. Microcystin quota, cell division and microcystin net production of precultured *Microcystis aeruginosa* CYA 228 (Chroococcales, Cyanophyceae) under field conditions. Phycologia 42, 667-674.

Marwood, C. A., Solomon, K. R., Greenberg, B. M., 2001. Chlorophyll fluorescence as a bioindicator of effects on growth in aquatic macrophytes from mixtures of polyciclic aromatic hydrocarbons. Environ. Toxicol. Chem. 20, 890-898.

Mikalsen, B., Boison, G., Skulberg, O. M., Fastner, J., Davies, W., Gabrielsen, T. M., Rudi, K., and Jakobsen, K. S., 2003. Natural variation in the microcystin synthetase operon *mcy*ABC and impact on microcystin production in *Microcystis* strains. J. Bacteriol. 185, 2774-2785.

Nilsson, G., 1977. Effects of glyphosate on the amino acid content in spring wheat plants. Swedish J. Agric. Res. 7, 153-157.

NIVA, 1990. Research with algal cultures- NIVA's Culture Collection of Algae. Norwegian Institute for Water Research, Oslo. 82-577-1743-6.

OECD, 2006. Test guideline 201. Freshwater alga and cyanobacteria, growth inhibition test. Organization for Economic Co-operation and Development, Paris.

Oh, H.-M., Lee, S. J., Jang, M.-H., and Yoon, B.-D., 2000. Microcystin production by *Microcystis aeruginosa* in a phosphorus-limited chemostat. Appl. Environ. Microbiol. 66, 176-179.

Orr, P. T., and Jones, G. J., 1998. Relationship between microcystin production and cell division rates in nitrogen-limited *Microcystis aeruginosa* cultures. Limnol. Oceanogr. 43, 1604-1614.

Paerl, H. W., 1988. Growth and reproductive strategies of freshwater blue-green algae (cyanobacteria). In Sandgren, C. D. (Eds.), Growth andreproductive strategies of freshwater phytoplankton, Cambridge University Press, Cambridge, pp. 261-315.

Pearson, L. A., Hisbergues, M., Börner, T., Dittmann, E., and Neilan, B. A., 2004. Inactivation of an ABC transporter gene, *mcyH*, results in loss of microcystin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806. Appl. Environ. Microbiol. 70, 6370-6378.

Powell, H. A., Kerby, N. W., and Rowell, P., 1991. Natural tolerance of cyanobacteria to the herbicide glyphosate. New Phytol. 119, 421- 426.

Powers, C. D., Wurster, C. F., and Rowland, R. G., 1979. DDE inhibition of marine algal cell division and photosynthesis per cell. Pest. Biochem. Physiol. 10, 306-312.

Rapala, J., Sivonen, K., Lyra, C., and Niemelä, S. I., 1997. Variation of microcystins, cyanobacterial hepatotoxins, in *Anabaena spp*. as a function of growth stimuli. Appl. Environ. Microbiol. 63, 2206-2212.

Ravi, V., and Balakumar, T., 1998. Biodegradation of the C-P bound in glyphosate by the cyanobacterium *Anabaena variabilis* L. J. Sci. Ind. Res. (India) 57, 790-794.

Ross, C., Santiago-Vásquez, L., and Paul, V., 2006. Toxin release in response to oxidative stress and programmed cell death in the cyanobacterium *Microcystis aeruginosa*. Aquatic Toxicol. 78, 66-73.

Shubert, L. E., 1984. Algae as ecological indicators. Academic Press, London.

Sibly, R. M., Hone J., 2002. Population growth rate and its determinants: an overview. Phil. Trans. R. Soc. Lond. B 357, 1153-1170.

Smith, E. A., and Oehme, F. W., 1992. The biological activity of glyphosate to plants and animals: a literature review. Vet. Human Toxicol. 34, 531-542.

Solomon, K. R., and Thompson, D. G., 2003. Ecological risk-assessment for aquatic organisms from over-water uses of glyphosate. J. Toxicol. Environ. Health, Part B 6, 289-324.

Tillet, D., Dittmann, E., Erhard, M., Von Döhren, H., Börner, T., and Neilan, B. A., 2000. Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-poliketide synthetase system. Chem. Biol. 7, 753-764.

USEPA, 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. United States Environmental Protection Agency, Washington.

Utkilen, H., and Gjølme, N., 1995. Iron-stimulated toxin production in *Microcystis aeruginosa*. Appl. Environ. Microbiol. 61, 797-800.

Verschueren, K., 1983. The Handbook of Environmental Data on Organic Chemicals. Van Nostrand Reinhold, New York.

Wang, J.-X., Xie, P., and Guo, N., 2007. Effects of nonylphenol on the growth and microcystin production of *Microcystis* strains. Environ. Res. 103, 70-78.

Wängberg, S.-Å., and Blanck, H., 1988. Multivariate patterns of algal sensitivity to chemicals in relation to phylogeny. Ecotoxicol. Environ. Safety 16, 72-82.

Ward, C. J., and Codd, G. A., 1999. Comparative toxicity of four microcystins of different hydrophobicities to the protozoan, *Tetrahymena pyriformis*. J. Appl. Microbiol. 86, 874-882.

Costs and benefits of toxin production in the cyanobacteria *Microcystis aeruginosa*

Katia Montenegro[a,b], and Göran Bengtsson[a]
[a]Department of Ecology, Lund University, Sölvegatan 37, SE-22362 Lund, Sweden
[b]CIRA-UNAN, Aptdo. Postal 4598, Managua, Nicaragua

Abstract

Organisms allocate assimilated resources into competing metabolic demands, such as maintenance, growth and defense. This pattern of allocation may change in response to stress and result in an increment of resources allocated to one trait and a decrement to another trait. For example, there should be a trade-off between capacity to survive and growth rate as a response to starvation. The aim of the study was to test for the existence and magnitude of a potential trade-off between growth and toxin production in *M. aeruginosa* exposed to the herbicide glyphosate at different light regimes and phosphorus concentrations. The ecological and metabolic costs of microcystin production were estimated from growth rates and net energy costs in a wild type strain of *M. aeruginosa* and its mutant unable to produce microcystins. The trade-off was only detected when cultures were incubated under photoperiods longer than 8 h of light (12:12 and 16:8), with differential patterns per microcystin analog, time of incubation and exposure concentration. Growth was the definitively favoured trait during early exponential growth phase, and less favoured over toxin production during the late. Glyphosate could not invert the nature of the interaction between both traits along with an impressive reduction of its herbicidal action on the cyanobacterial cells in the phosphorus experiments where cells were pre-P-starved, or when the cultures were incubated under the shortest photoperiod of 8 h of light. The metabolic and ecological costs involved in microcystin production are higher during the first week of incubation and minimized afterwards after comparison between cell quotas of ATP and growth rates in the wild type and the mutant cultures. Glyphosate conferred additional ecological advantages to the mutants expressed in terms of tolerance, and especially when dosed at the highest tested concentration (40 mg/L). Glyphosate, however, seem to confer benefits to the exposed cultures in the second week of incubation by either increasing toxin production or inhibiting the extracellular releases to the medium. In general, the production of the most toxic analog MC–LR seem to be favoured over the one of [D–Asp^3]MC–LR in the first seven days of incubation, and such favoritism minimized in the last week of incubation when both productions tended to be similar or slightly higher for [D–Asp^3]MC–LR. The negative production rates calculated for [D–Asp^3]MC–LR, during the first week of incubation in the light experiments might imply that the [D–Asp^3]MC–LR pool is not stable and that the compound is synthesized, used, and replenished constantly under the given culturing conditions. This turnover of the analog seems to be triggered by glyphosate when the cultures were incubated under the 12:12 photoperiod (15 and especially 50 mg/L), under the longest photoperiod (16:8, all treatments), and genetically fixed under a dim light photoperiod (8:16).

Introduction

Organisms allocate assimilated resources into competing metabolic demands, such as maintenance, growth, reproduction, and defence (Townsend and Calow, 1981; Congdon et al., 2001). If internal resources are limited and insufficient to pay all construction and maintenance costs for two life history traits that share a common resource pool, then a trade-off results: an increment of resources allocated to one trait necessitates a decrement of resources to another trait (Zera and Harsham, 2001). For example, organisms may respond to stress, such as starvation, by trade-offs between survival, growth and reproduction (Calow, 1991) following priority rules shaped by ecological factors (Zera and Harsham, 2001).

Trade-offs have been examined in various ecological contexts, such as defence against herbivory (Benjamin and Klaine, 1995; Yoshida et al., 2004) and often quantified as an inverse correlation between growth or reproduction and the levels of toxins (Benjamin and Klaine, 1995; Steinberg, 1995; Pintar and Starmer, 2003; Yoshida et al., 2004; Donaldson et al., 2006). This might imply that a negative functional interaction is the cause of the negative association between the traits under consideration (Zera and Harsham, 2001). Trade-offs are dynamic and may vary with species and life-history stages, if patterns of allocation are constrained by endogenous (genetically fixed) metabolic or developmental cycles, so the same defences may be costly in some species, or at some times or places, but not in others (Steinberg, 1995). Benefits are expected to outweigh costs of trade-offs by increasing some component of fitness in the trader organism (Pintar and Starmer, 2003; Donaldson et al., 2006).

Micro-algae (e.g., *Chlorella vulgaris* and *Scenedesmus* spp.) show trade-offs between growth and grazing-resistance (Lürling and Van Donk, 2000; Yoshida et al., 2004), and others (e.g., *Selenastrum capricornutum* adaptation to Cu pollution) between resistance/tolerance to combat an anthropogenic stress and growth (Benjamin and Klaine, 1995). Trade-offs have received relatively little attention in ecotoxicological testing (Benjamin and Klaine, 1995), in spite of the knowledge that critical effect levels for sensitive life cycle traits are not sufficient for assessing the potential impact of toxicants on fitness, since given that each single trait has its own plasticity and a differential relationship with fitness depending on the life history strategy of the organism (Kammenga et al., 1997).

Cyanobacterial genuses, such as *Microcystis* and *Anabaena*, are classified as resource-(*K*)-selected species (Reynolds, 2006) or at least to be placed along the *r*- through *K*-selection continuum of life-history strategies (Kilham and Hecky, 1988) and may be candidates for detectable trade-offs involving growth because they allocate resources to a variety of non reproductive activities that maximize survival of individuals, including abilities to obtain and store resources and adaptations that reduce mortality (e.g., manufacture of toxins) (Kilham and Hecky, 1988). Microcystins (MCs) are a group of at least 70 identified variants of cyclic heptapeptides, including methylated and demethylated products, which are produced by cyanobacterial genera predominantly including *Microcystis*, *Anabaena*, and *Planktothrix*, which also have non-microcystin producing members (Chorus and Bartram, 1999). Phylogenetic studies indicate that microcystin synthethase genes were present in the last common ancestor of a large number of cyanobacteria, and that the

ability to produce the toxins has been repeatedly lost during evolution, explaining the variability in toxicity encountered in natural populations (Dittmann and Börner, 2005).

The microcystin biosynthesis occurs non-ribosomally by a thio-template mechanism at expenses of ATP *via* a multifunctional enzyme complex, including peptide synthetase and polyketide synthese modules that accomplish, for example, 48 sequential catalytic reactions to produce one of the most toxic analogs, the MC–LR (Arment, 1996; Dittmann et al., 1997; Tillet et al., 2000). Metabolic costs related to microcystin production have been assumed to be high, since both large enzyme complexes and nitrogen-rich peptides are produced (Repka, 2004), but they are not quantified to our knowledge. Microcystins seem to make their producer competitively inferior under nutrient limiting conditions when non-microcystin producing *M. aeruginosa* grow better than microcystin-producing (Vézie et al., 2002; Yoshida et al., 2007).

Most microbial non-ribosomal peptides are classified as secondary metabolites and microcystins have long be regarded as such (Carmichael, 1992; Chorus and Bartram, 1999), but may also play a physiological role within the cell (e.g., quorum sensing agents, light harvesters or adapters, trans-membrane transporters or chelators of metals) (Utkilen and Gjølme, 1995; Dittmann et al., 2001; Hesse et al., 2001). Although several roles for microcystins are possible, their potential ecological or metabolic significance remains to be confirmed (Young et al., 2005).

The herbicide glyphosate is known to trigger molecular events that could account for growth inhibition in exposed organisms, including starvation for aromatic amino acids and energy drain (of ATP) which in turns must initiate other metabolic vulnerabilities (Fischer et al., 1986). Low concentrations of glyphosate reduce growth at the same time as the MC–LR production increases in *M. aeruginosa* (Paper I). Light limitation is typically assumed to be the most important stress for growth in primary producers, but nutrient limitation, especially of phosphorus, is perhaps the most often cited growth-limiting element in freshwater habitats and often reported as equally important (Paerl, 1988; Donaldson et al., 2006). This together with scarcity of studies on trade-offs between ecological factors (e.g., light or nutrient limitation) and xenobiotics motivated us to test for potential trade-offs in cyanobacteria.

The aim of the study was to test for the existence and magnitude of a potential trade-off between growth and toxin production in *Microcystis aeruginosa* exposed to the herbicide glyphosate at different light regimes and phosphorus concentrations. The ecological and metabolic costs of microcystin production were estimated from growth rates and net energy costs in a wild type strain of *M. aeruginosa* and its mutant unable to produce microcystins.

We hypothesized that:

i. The ratio between rates of growth and toxin production in a control culture would be higher than in glyphosate-exposed cultures because the herbicide would favour toxin production over growth when dosed at low to moderate concentrations. However, at the highest dosed concentration, growth would be definitively favoured over toxin production after a severe inhibition by the herbicide.

ii. The magnitude of the trade-off would be modulated by the co-effect of light and phosphorus, such that the growth/toxin production ratio should decrease with photoperiod and phosphorus concentration, ameliorating the effects of the herbicide on the trade-off. Light was assumed to be more influential as driving-force for the trade-off by directly increasing the amount of *mcy* transcripts per cell (microcystin synthetase genes) (Kaebernick et al., 2000), hence greatly influencing microcystin production rates, while phosphorus is expected to affect them only indirectly through its effect on growth and cell division (Orr and Jones, 1998).

iii. Glyphosate was assumed to be more deleterious for growth in the wild type strain than for its mutant since the mutant has no cost of maintaining microcystin production but can use the resources to lessen the herbicide effect.

2. Materials and Methods

2.1 Test species

The wild type, microcystin-producing strain, *Microcystis aeruginosa* (Kützing) Lemmermann PCC 7806 and its microcystin deficient mutant PCC 7806 Δ*mcyB* were kindly provided by Dr. Elke Dittmann, Institute for Biology, Humboldt University, Berlin, Germany. The wild strain produces mainly the microcystin variants MC–LR and [D–Asp^3]MC–LR and was originally isolated from Braakman Reservoir, The Netherlands (Mikalsen et al., 2003). The stock cultures of both strains were grown semi-continuously in sterile modified Z8 medium (Paper I) in 2 L Erlenmeyer flasks at ~ 20 ºC and under ~ 7–12 μmol photons/m^2 of cool white fluorescent light from 30 W Sylvania Standard® lamps (Germany) during 20 h/day.

2.2 Test chemicals

Glyphosate (IUPAC name: N-[phosphonomethyl]glycine) is a broad-spectrum, systemic herbicide, which acts by inhibition of the 5-enolpyruvylshikimic acid-3-phosphate (EPSP) synthase, a mid-pathway enzyme of the aromatic amino acid biosynthesis, which links primary and secondary metabolism in prokaryotes, lower eukaryotes, and higher plants (Smith and Oehme, 1992). Glyphosate (95% purity) was purchased from Sigma Chemical (St. Louis, MO, USA), and the stock solution (~ 5000 mg L^{-1}) was prepared in ultrapure water. Three nominal concentrations were prepared for the experimental batches, 1.5 mg/L (C1) the Environmental Expected Concentration for aquatic exposure (EEC) (Battaglin et al., 2005), 15 mg/L (C2), a concentration known to affect the endpoints of interest in *M. aeruginosa* strains, and 40 mg/L (C3), a concentration known to have profound effects (Paper I).

2.3 Experimental design

Aliquots of semi-continuous cultures of the PCC 7806 and its mutant were inoculated in glass jars with modified Z8 growth medium, either complete or P–depleted (phosphorus experiments), to obtain batch cultures with an initial chlorophyll

a (Chl *a*) concentration of about 100 µg/L (6–7×10^8 cells/L). The experiments were performed in a temperature controlled room at 20±1°C and under 1–4 µmol photons/m^2 provided by two 30W sunlight lamps (Sun-glo® and Flora-glo®, Japan) at different photoperiods depending of the experiment (see below). Five (light experiments, see below) or three (P experiments, see below) replicates per strain were prepared in uncontaminated growth medium (control), and the same number of replicates were prepared per each glyphosate concentration tested. Samples were taken after 0, 7 (middle exponential growth phase), and 14 days (late exponential growth phase) for cell counting (all replicates), intracellular toxin content (all PCC 7806 replicates), and total ATP content, from three replicates in the light experiments and from all replicates in the P experiments. The persistence of the mutation in the batch cultures of PCC 7806 Δ*mcy*B was verified by undetectable amounts of intracellular MCs at the start of each bioassay. One replicate per concentration was usually analyzed for MCs content in water at all sampling occasions, and two replicates at 7 and 14 days for the light experiments. The jars were covered with parafilm to allow some gas exchange and light penetration but prevent from contamination, and repositioned randomly each day. Parallel control culture sets of two replicates of both strains were sampled for cell counting and Chl *a* content every third day up to 15 days. The Chl *a* content was extracted according to Jespersen and Christoffersen (1987) and quantified in an ultraviolet–visible light spectrophotometer Ultrospec 3000 (Pharmacia Biotech, Cambridge, England).

The light experiments were performed at three diel photoperiods (h light: h dark cycles): 12:12 (batch I), 16:8 (batch II), and 8:16 (batch III) to mimic light conditions in tropical countries (12:12), and extremes in temperate countries during summer (18:6), and winter (8:16). The phosphorus experiments (batch IV) were performed in complete Z8 medium (Z8+ ≈ 5.4 mg/L P), partially P–depleted Z8 medium (1/2 of the original concentration, Z8½ ≈ 2.7 mg/L P), and completely P–depleted Z8 medium (Z8-) under a 12:12 photoperiod. The stock cultures were P–starved by addition of Z8- medium for at least one week prior to the experiments. The complete and partially P–depleted Z8 media were amended with equi-molar concentrations of potassium by replacing K_2HPO_4 with KCl (Repka, 2004).

2.4 Growth rates

Specific growth rates were estimated from logarithmic changes in cell density over 7 day periods, assuming first order kinetics (i.e., the rate of increase in cells per unit time is proportional to the number of cells present in the culture at the beginning of any unit of time), and expressed on per day basis (day^{-1}) (Orr and Jones, 1998; Wood et al., 2005). The samples for counting were preserved by addition of a 50 % solution of glutaraldehyde (Sigma-Aldrich Chemie, GmbH, Steinheim, Germany) at 2 % final concentration and refrigerated at 4 ºC in the dark until analysis (< two weeks). One mL aliquots of preserved *M. aeruginosa* cells were counted using a FACSort flow cytometer (Becton-Dickinson Instruments, San José, CA, USA) equipped with a 15-mW argon-ion laser (488-nm). The autofluorescence of Chl *a* was measured in the red fluorescence channel (FL3), and the autofluorescence of phycoerythrin in the orange fluorescence channel (FL2). The forward angle light scatter (FSC) signal was used as the acquisition threshold (i.e., trigger signal). The best regions discrimination was obtained using a dual gating procedure based on two-dimensional cytograms of FSC versus FL3 and FSC versus FL2. However, since chlorophyll fluorescence usually gave higher % gating of events (> 90) than phycoerythrin, it was the selected

signal used for calculations and as proxy for the physiological state of the cells that were usually divided in two sub-populations. The counts were performed at a medium flow rate (35 µL ± 5 µL/min) with events acquisition over 30 s. The absolute cell counts per mL were obtained by multiplying the total counts from regions of interest initially created around control wild-type PPC 7806 and the mutant by the dilution factor and then dividing by the volume counted in 30 s that was calculated from calibrated flow rates that were measured when possible by the gravimetric method (Marie et al., 2005). Growth rates based on the Chl content and cell counts were compared in two replicates of controls and treatments in the light experiments.

2.5 Microcystin analysis

Intracellular MCs were extracted as described in Paper I, with the exception that a fixed volume of 100 mL of sample was filtered. MCs dissolved in water were concentrated on 3M Empore™ C_{18} extraction disks (St. Paul, MN, USA). These samples were prepared with a modified procedure of the one recommended by (Rivasseau et al., 1998) to optimize MCs extraction and processed within one week after sampling and kept refrigerated until analysis. Three hundred mL of pre-filtered sample (pooled liquid from three intracellular MCs samples) were supplemented with TFA (trifluoroacetic acid, Sigma-Aldrich Chemie) and methanol (Fluka HPLC grade, Sigma-Aldrich Chemie) to obtain 0.1 % and 5 % final concentrations. The discs were conditioned according to the manufacturer's instructions, and the supplemented sample was added, allowing enough contact time with the solid phase and avoiding bubble formation. When the sample was completely percolated, 10 mL of 30 % aqueous methanol were added to clean the disk. Then vacuum was applied for 15 min to remove as much residual water as possible. MCs were subsequently desorbed with 3×10 mL acidified methanol (0.1 % TFA), each aliquot was evaporated and pooled until dryness in a vacuum centrifuge (SC110A SpeedVac®Plus, Savant Instruments Inc., Ohio, USA) at a medium drying rate (~ 40 ºC). The rest of the procedure was identical to the one described in Paper I, and made under the same conditions with the same equipments, except for the volume resuspension that was changed to 100 µL of methanol.

The separation and quantification of MC–LR and [D–Asp^3]–LR was made by reversed phase in a Hewlett Packard HP Series 1050 HPLC (Hewlett Packard GmbH, Waldbronn, Germany) under the same conditions and with the same equipment as described in Paper I for the pp'-DDE experiment, but with the eluent acidified with 0.05 % TFA. The MC concentrations were quantified from average daily response factors of at least two standards of MC–LR (Calbiochem, La Jolla, CA, USA) in the range from 1 to 40 ng/µl (1–5 for water, and 10–40 for intracellular). The response factor of [D–Asp^3]–LR was assumed to be the same as for the methylated variant (Orr and Jones, 1998) given the absence of a standard. The injection volume was usually 10 µl for intracellular MCs and 20 µl for MCs in water. The specific toxin production rates were estimated from logarithmic changes in the total microcystin content per volume (intra + extracellular content) over 7 day periods, assuming first order kinetics (i.e., the rate of increase in toxin content per unit time is proportional to the toxin concentration at the beginning of any unit of time), and expressed in per day basis (day^{-1}) (Orr and Jones, 1998).

Further identification of the microcystin variants was made using a triple quadrupole mass spectrometer (MS) with electrospray ionization (ESI) on a turbo

ionspray source (API 3000, Applied Biosystems, Foster City, CA, USA) coupled to a liquid chromatography system from Perkin Elmer (Norwalk, CT, USA; LC-MS/MS). Three µL of sample extract were injected on a 150×3 mm C18 Luna 5 µm (2) (Phenomenex Inc., CA, USA) column protected by a Phenomenex C18, 4×2 mm guard column. The mobile phase consisted of water and methanol, both containing 0.5 % acetic acid. The separation was carried out using a linear gradient, starting from 5 % to 95 % methanol in 20 min, followed by 1 min isocratic flow at 95% methanol at a flow rate of 0.5 mL/min. The [D–Asp^3]–LR was detected at m/z 981.7, and MC–LR at m/z 995.7. The temperature of the auxiliary gas was set to 350°C and the ion spray voltage was 5000 V. Nitrogen was used as the curtain, turbo ionspray, collision, and nebulizer gas.

2.6 ATP measurement

The total cellular ATP (adenosine triphosphate) measurements were performed using the ENLITEN® rLuciferase/Luciferin (L/L) reagent (Promega, Madison, WI, USA) for bioluminescence detection at the sampling day. The photons produced by the reaction were counted with a Beckman LS 6500 Multipurpose scintillation counter (Fullerton, CA, USA) under the single photon option in manual mode, and cpm (counts per minute) were averaged for 5×5 s (25 s) after a 30 s delay. The samples were first extracted to lyse cells and inactivate ATPases by combining 150–200 µl of cells suspension with the same volume of ultrapure water preheated to 100 ºC in sterilized Eppendorf tubes, and boiling for 5 min with occasional vortexing (Napolitano and Shain, 2005)(Napolitano and Shain 2005). Extracted samples were kept on ice until analysis (< 1 h). Bioluminescence was monitored when 100 µl of reconstituted L/L reagent were gently pipetted to the bottom of sterilized 6 mL plastic scintillation vials and then 150 µl of extracted sample or an aliquot diluted to that volume were added, ensuring a good homogenization but avoiding violent mixing. A reagent blank, containing L/L reagent, the sample medium and extractant, was run each time the analysis was made to determine the amount of background luminosity to be subtracted from the samples. The net luminosity counted in the samples was usually in the range of 10^6 to 10^7 cpm and with a coefficient of variation of 1–2 %. At least five standard solutions in the range from 1×10^{-11} to 8×10^{-10} M (5–400 ng/L), including a blank, were prepared with ultrapure water by serial dilution of the resuspended stock standard ~ 1.2 mg of $Na_2ATP\cdot3H_2O$ equivalent to ~ 1 mg of ATP (~ 2 µM) (Sigma-Aldrich Chemie). The volumetric ATP content of the samples was obtained directly from extrapolation of their luminescence against the linear standard curve of the day, with subsequent multiplication with the dilution factor.

2.7 Data calculation and analysis

Microcystins and ATP concentrations were calculated on culture volume basis (e.g., µg MC–LR per litre) and as cell quotas (e.g., fg MC–LR per cell), since the latter is recommended to reflect true variations, for example in microcystin content (Orr and Jones, 1998). The percentage inhibition or stimulation of specific growth or toxin production rates at each test concentration was calculated as described in Paper I. Ln-transformed data of cell densities, MC–LR, [D–Asp^3]–LR, and ATP concentrations and quotas were analyzed with a mixed model of RM-ANOVAs (repeated–measures analysis of variance) within replicates throughout time of incubation (0, 7 and 14 days) or within strains (mutant and wild type) or toxins ([D–Asp^3]–LR and MC–LR), and between groups (controls and treatments). Simple contrasts were conducted to compare treatments with controls, strains or toxins, while

repeated contrasts were conducted to compare results from 0 to 7 days, and from 7 to 14 days. Data normality, homogeneity of variances, and sphericity were evaluated with Shapiro-Wilk's, Levene's, and Mauchly's tests, respectively. The results of the mixed statistical model were adjusted when sphericity was violated by selecting the Greenhouse-Geisser statistics to report significance of the differences in the tested factors, and by using the Bonferroni method to adjust means for multiple comparisons between groups. Statistics were performed using SPSS for Windows 9.0 (Chicago, IL, USA) at a significance level of 5 % (p=0.05).

3. Results

3.1 Growth vs. microcystin production (the potential trade-off)

The ratios between average specific growth rates (Appendix, Table 1) and average specific production rates of MC–LR and [D–Asp^3]MC–LR (Appendix, Table 2) that were calculated to measure the magnitude and nature of the functional interaction (positive or negative) between the two traits are presented in Table 3.

3.1.1 Light experiments

Growth was in general favoured over MC–LR production (10 %–9×), especially when the cultures were incubated under the shorter photoperiod (8:16). Growth was relatively more promoted by variations in the light regime than by variations in P concentrations, which had marginal influence on the effect of glyphosate on the μ/MC–LR ratio, at least during the firs 7 days. The ratios of the treatments were higher than those of the controls and increased in response to the glyphosate dose during the first 7 days of incubation. Glyphosate when dosed at C3 was curiously not detrimental for growth when cultures were incubated under the shortest photoperiod (8:16) in the first 7 days of incubation, and as expected, less detrimental under a longer photoperiod (16:8) given that the rates were positive, while MC–LR production was negative. The C3 treatments incubated under a photoperiod 12:12 presented negative rates for both traits, but still less negative rates for growth than for production.

The cultures grew faster than they produced [D–Asp^3]MC–LR (10 %–19×), especially during the first 7 days of incubation. All cultures in the low light (8:16) had negative rates during the first 7 days of incubation. The ratios generally became lower in the second incubation week. There were no consistent trends in ratios in response to the glyphosate dose.

More MC–LR was produced than [D–Asp^3]MC–LR (2–15×) in the first seven days of incubation (Table 2), especially in the control cultures incubated under the longest photoperiod (16:8) that had positive rates for both analogs, and least in the C3 treatments incubated under a photoperiod (12:12) where both analogs had negative rates. However, after 14 days, the rates of both microcystins tended to be quite similar.

3.1.2 Phosphorus experiments

Growth was in general less favoured over MC–LR production (10–80 %) than in the light experiments, and all ratios were within a range of 0.5–2.0 with no obvious dependence on P or glyphosate concentrations.

Table 3. Ratios between average specific growth rates (μ, day^{-1}) and average specific production rates of MC–LR and [D–Asp^3]MC–LR (day^{-1}) in control batch cultures of *Microcystis aeruginosa* PCC 7806 and glyphosate treatments (C1=1.5, C2=15, and C3=40 mg/L) that were incubated during 14 days. The rates were derived from 7 days logarithmic changes in cell density and bulk volumetric microcystin concentration (intra + extracellular, μg/L) of three (batch IV) and five replicates (other batches). Negative values imply that growth rate was negative, underlined that production rate was negative, and **bold** that both rates were negative. The light experiments were performed under different photoperiods h light: h darkness, and in the phosphorus experiments Z8: no P, Z8½ ~ 2.7 mg/L P, and Z8+ ~ 5.4 mg/L P.

Batch/ Groups	μ/MC–LR production		μ/[D–Asp^3]MC–LR production	
	0–7 d	7–14 d	0–7 d	7–14 d
12:12 Light				
Control	0.7	2.7	2.8	2.3
C1	1.1	1.9	5.4	2.0
C2	1.2	1.6	8.3	1.5
C3	**2.5**	-2.7	**4.8**	-2.0
16:8 Light				
Control	1.3	2.2	19.0	1.3
C1	1.5	1.9	4.2	1.0
C2	1.8	1.2	2.5	0.9
C3	5.0	1.2	12.0	1.1
8:16 Light				
Control	4.5	1.1	2.2	1.2
C1	4.6	1.5	2.3	1.5
C2	8.5	1.7	2.4	2.2
C3	9.1	1.8	2.0	2.3
Z8- Phosphorus				
Control	0.9	0.9	1.4	0.8
C1	0.7	1.4	1.1	1.1
C2	1.0	1.3	1.3	1.3
C3	1.1	1.0	3.2	1.0
Z8½ Phosphorus				
Control	1.0	0.8	1.7	0.6
C1	1.2	1.1	2.1	0.5
C2	1.1	1.8	2.0	1.4
C3	1.1	1.4	5.5	1.2
Z8+ Phosphorus				
Control	1.3	1.2	3.6	0.9
C1	0.8	1.7	1.5	1.1
C2	1.3	1.2	2.4	1.1
C3	1.7	1.4	4.0	1.5

The [D–Asp^3]MC–LR production was more disfavoured over growth than MC–LR (10 %–5.5×) especially during the first incubation week and at the highest glyphosate concentration.

More MC–LR than [D–Asp^3]MC–LR again (30 %–5×) was produced during the first incubation week, especially at the highest glyphosate concentration (Table 2), but less or equally much during the second week, regardless of the P concentration.

3.2 Light *vs*. phosphorus (importance as triggers of the potential trade-off)

The importance of light *versus* phosphorus as trigger of the potential trade-off between growth and microcystin production was analyzed by comparing analogs (e.g., controls vs. controls) in the three light batches to discriminate the mere effect of light from the one of glyphosate, and in the three phosphorus batches to extract the mere effect of phosphorus.

3.2.1. Light

A longer photoperiod (16:8, second batch) supported higher growth rates (~ 20 %) than the reference photoperiod (12:12) notably in the first 7 days of incubation and significantly only for the C1 and C3 treatments (p=0.000–0.013). The shorter photoperiod (8:16) had significantly faster growth rates than the reference photoperiod (p=0.000–0.032), especially at the highest glyphosate concentration in the first seven days of incubation. The shorter photoperiod (8:16) had significantly lower (p=0.000–0.031) production rates for both analogs than the reference photoperiod (Table 2). Both analogs had higher production rates in the 16:8 photoperiod than in the reference photoperiod, but the production rates of the MC–LR analog were essentially the same in 12:12 and 16:8 during the first week.

3.2.2. Phosphorus

There were not significant differences in growth rates between the phosphorus levels (mixed model p=0.063–0.100) (Table 1). Both MC–LR analogs had the highest production rates in absence of phosphorus (Z8-), although the mixed model gave significant differences among the P groups only for the control and C2 treatments (p=0.004–0.035) (Table 2)

Some interesting patterns emerge when growth and toxin production rates are compared in the 12:12 assay (light experiments) and the Z8+ (phosphorus experiments) with the same incubation photoperiod but with a P-starved inoculum in the phosphorus bioassay (Appendix, Table 2). The P-starved cultures had higher growth rates (20 %–6×) in the first 7 days of incubation but lower during the second week, except for the cultures exposed to the highest glyphosate concentration, which generally grew far better when they were pre-starved for P.

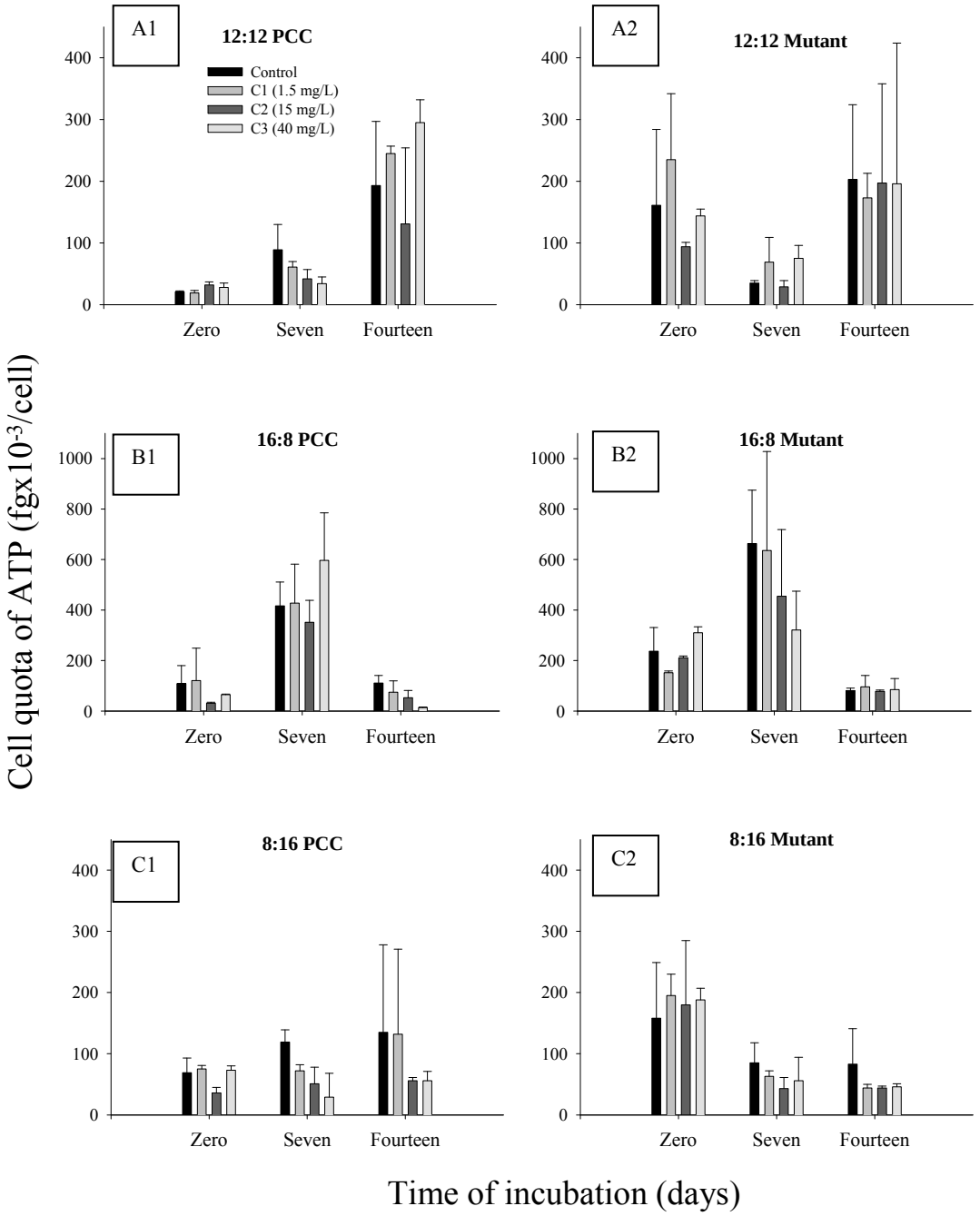

Figure 1. Time trends of average ATP cell quotas (fg×10^{-3}/cell) in batch cultures of *Microcystis aeruginosa* PCC 7806 and its mutant PCC 7806 Δ*mcy*B unable to produce microcystins. Control cultures and glyphosate treatments were grown under three diel photoperiods (h light: h dark cycles). Note the different scale in B1 and B2. The different bars represent treatments with glyphosate and a control and are averages of two (0 days), and three replicates (7 and 14 days), respectively. Error bars represent the fractional standard deviations of the means.

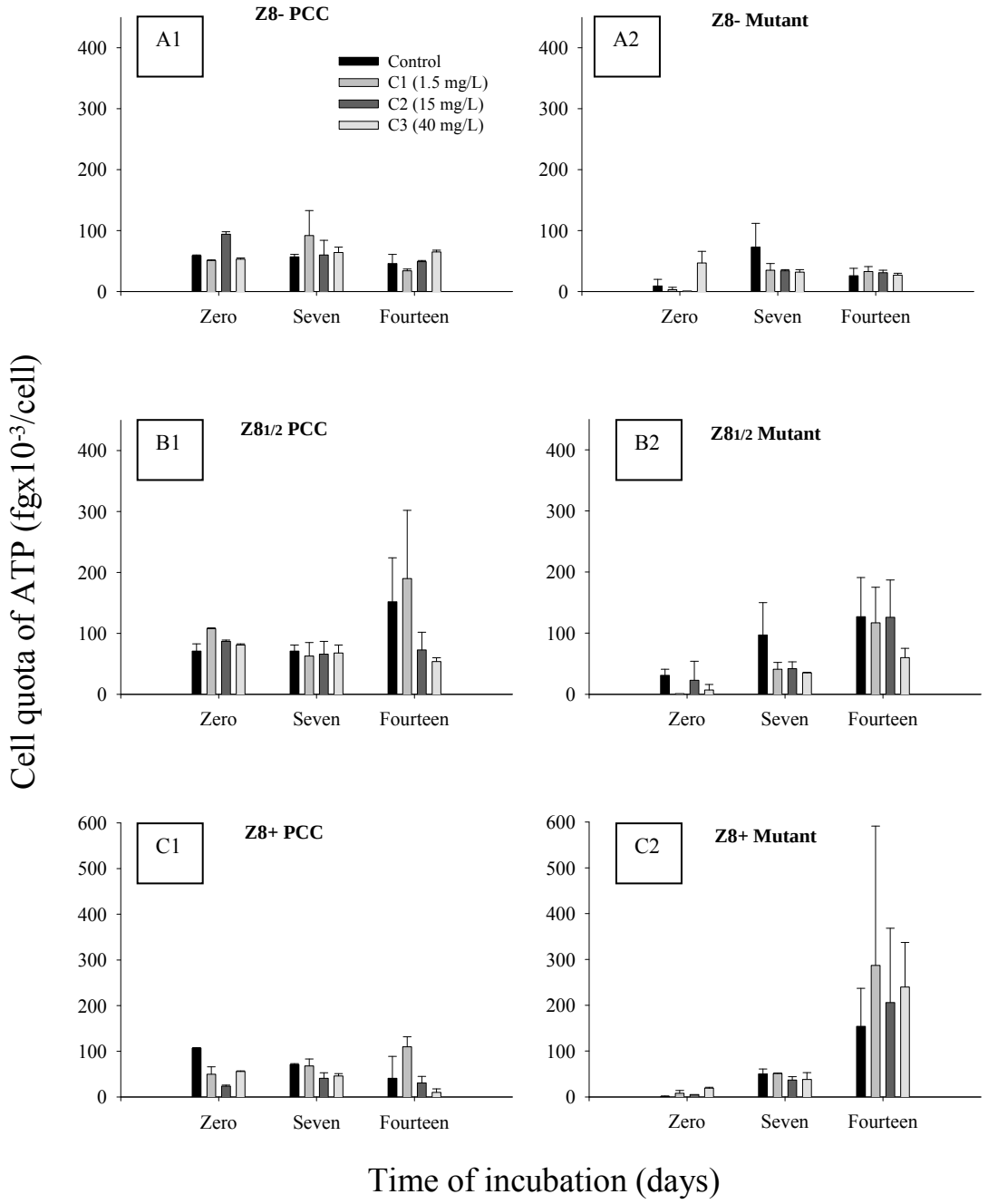

Figure 2. Time trends of average ATP cell quotas (fg×10^{-3}/cell) in pre-P-starved batch cultures of *Microcystis aeruginosa* PCC 7806 and its mutant PCC 7806 Δ*mcy*B unable to produce microcystins. Control cultures and glyphosate treatments were grown at three phosphorus concentrations (Z8: no P, Z8½ ~ 2.7 mg/L P, and Z8+ ~ 5.4 mg/L P) at a 12:12 photoperiod. Note the different scale in C1 and C2. The different bars represent treatments with glyphosate and a control and are averages of two (0 days), and three replicates (7 and 14 days), respectively. Error bars represent the fractional standard deviations of the means.

3.3 ATP cell quotas in the wild type strain *vs*. in the mutants (the metabolic costs)

The ATP cell quotas in the wild type cultures were compared with the quotas in the mutant, unable to produce microcystins, to estimate the potential metabolic costs of microcystin production (Figs. 1–2).

3.3.1 Light experiments

The cell quotas of ATP in the wild type strain (PCC) increased with incubation time in the 12:12 and 8:16 photoperiods with the cell quotas considerably lower in the 8:16 photoperiod after 14 days, especially in the glyphosate treatments (Fig. 1). The 16:8 photoperiod has a much higher cell quota yield than the other two photoperiods, with a peak after seven days of incubation. The same temporal pattern was repeated by the mutant in the 16:8 photoperiod, but the temporal pattern for the mutant in the other two photoperiods was almost a mirror of the pattern by the PCC strain. The cell quotas of the mutant were not significantly different in the controls and treatments (p=0.264–0.743), while for the PCC only significant in the 16:8 photoperiod (p=0.003) when cells were exposed to C2 (p=0.002) and C3 (p=0.002).

It was obvious that the mutants initially contained more ATP than the wild type cells, and the ratios between quotas differed by a factor of 1–7× and became < 1 after 7 and 14 days. The quotas of both strains were significantly different in the 12:12 and 16:8 photoperiods (p=0.001), and only marginally insignificant in the 8:16 (p=0.059). The interaction time × strain showed that the cell quotas of the strains were significantly different in the first week of incubation regardless of the photoperiod (p=0.000–0.024), but not in the second week (p=0.085–0.855).

3.3.2 Phosphorus experiments

There were not consistent trends in the temporal pattern of the cell quotas of ATP in the wild type strain (PCC) in the P experiment, except for the Z8½ cultures, in which the control and lowest glyphosate concentration gave cell quotas that were higher than any others (Fig. 2). This was in contrast to the temporal pattern for cell quotas in the mutant in presence of P, which showed an increase as the incubation proceeded. The overall univariate test for differences between groups was not significant for the PCC strain in the Z8- (p=0.181) and Z8½ (p=0.190) batches, but significant in the Z8+ when cells were exposed to C3 (p=0.020). All the mutant-treated cultures were significantly different to the controls in the Z8- batch (p=0.000), while only the C1 and C3 treatments were different in the Z8½ batch (p=0.001), and the C3 treatments in the Z8+ (p=0.015).

As opposed to the light experiments, the wild type cells initially contained more ATP than the mutant cells (2–14×). The quotas of both strains were significantly different in the Z8- and Z8½ batches (p=0.000), but not in the Z8+ (p=0.196). The ATP cell quotas between strains were highly significant different in the first week of incubation for the Z8- and Z8½ batches (p=0.000) but not significant in the second week (p=0.122–0.968), and highly significant along the Z8+ batch (p=0.000).

3.4 Growth in the wild type strain *vs*. in the mutants (ecological costs)

The potential ecological costs in terms of reduced intra-specific competitive ability and reduced tolerance to pollution for a microcystins producer compared to a

non-toxin producer was estimated by comparison of the growth rates of the wild type cultures and the mutant unable to produce microcystins (Appendix, Table 1).

3.4.1 Light experiments

The mutant grew generally twice as fast or faster than the wild type during the first week of incubation (ratio in Table 1), and the strains grew generally equally fast during the second week. Neither the photoperiod nor the glyphosate treatments had consistent influence on the growth ratio. This implies that the highest ecological costs were paid by the wild type cells in the first week of incubation. Glyphosate conferred immense advantages to the mutants when dosed at C3 in the cultures incubated under the longest photoperiod (22×), important when dosed at C2 for the cultures incubated under the three photoperiods (2.6–3.2×), and moderate when dosed at C1 (1.6–2×). The strains had significantly different growth rates (p=0.000–0.004) in the three batches, while their differences in time were significant along the 12:12 bioassay (p=0.000) and the 8:16 bioassay (p=0.000–0.034), and only during the first week for the 16:8 photoperiod (p=0.000).

3.4.2 Phosphorus experiments

The mutant grew 1.5–3× faster than the wild type during the first incubation week (ratios in Table 1), regardless of photoperiod or glyphosate concentration. During the second week, the two strains grew with the same rate, or the wild type grew slightly faster (Z8+). This implies that the highest ecological costs were paid by the wild type cells in the first week of incubation. Glyphosate conferred important advantages to the mutants when dosed at C3 (1.9–2.7×) least in the Z8+ batch, and moderate (1.5–2×) when dosed at C1 (least in the Z8½) and C2 (least in the Z8- batch). However, the strains did not have significantly different growth rates in the three bioassays (p=0.142–0.916), especially in the Z8+.

3.5 Intracellular cell quotas and extracellular concentrations of microcystins (the benefits)

3.5.1 Light experiments

The total intracellular cell quotas of MCs were of about the same magnitude regardless of the photoperiod (Fig. 3). The quotas in the 12:12 photoperiod assay peaked after 7 days, with one exception, the highest glyphosate concentration, for which the quotas were highest after 14 days and for the whole experiment. The 16:8 and 8:16 photoperiod assays had a descending temporal pattern in controls and treatments. The effects of glyphosate were significant only for the C3 treatments of the 12:12 assay for both MC–LR and [D–Asp^3]MC–LR (p=0.000 and p=0.005, respectively) and along the batch. MC–LR was the main contributor to the microcystins pool (60–75 %) along the bioassays, and with a tendency to slightly increase its contribution with time of incubation.

The highest extracellular microcystin concentrations found were detected at the beginning of the bioassays (20–30 % of the total pool), and the low light photoperiod (8:16) seemed to have the highest microcystin concentration at the beginning and the lowest after 7 and 14 days of incubation in spite of the only minor levels of MCs were detected in the medium (< 1 %) (Figs. 5A–C).

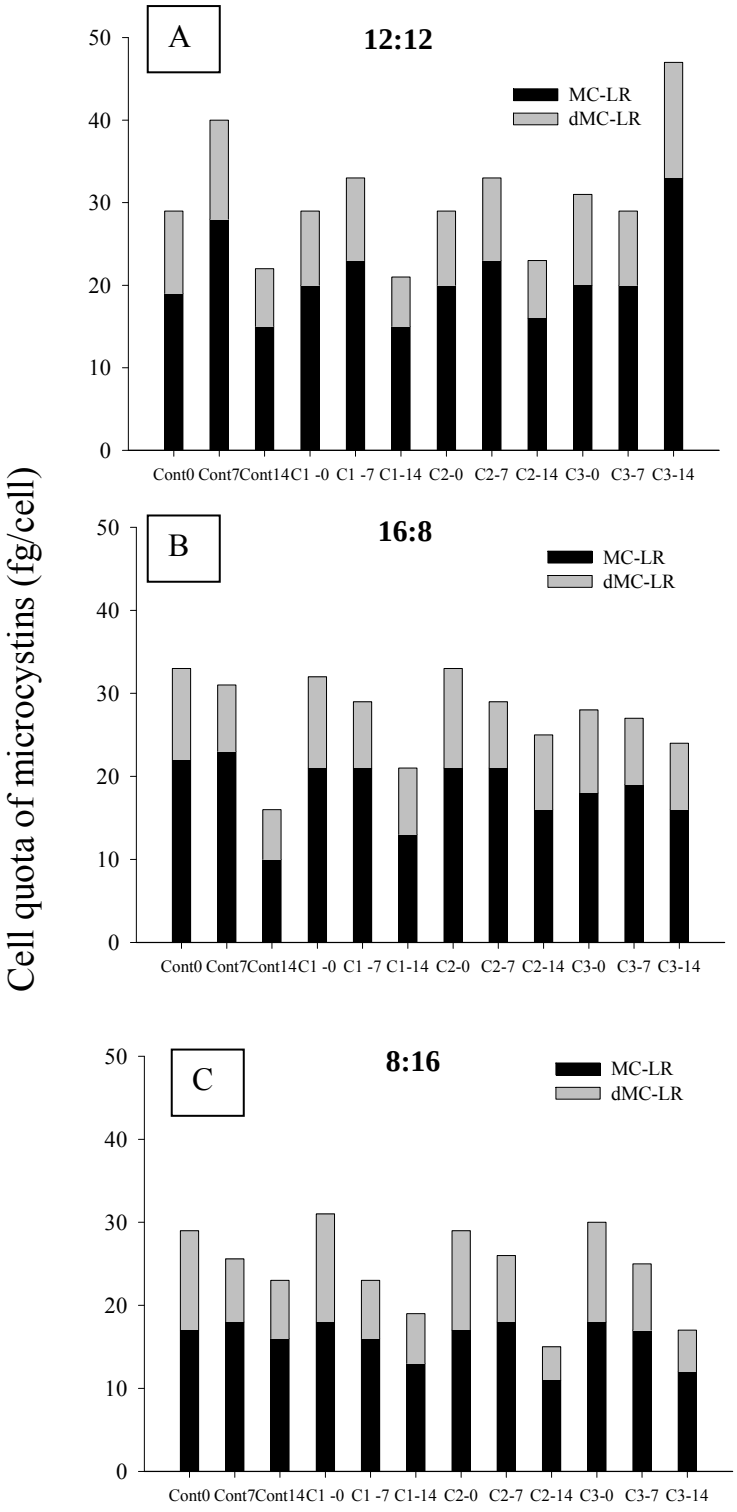

Figure 3. Time trends in average cell quotas of intracellular microcystins MC-LR and [D–Asp^3]MC–LR (fg/cell) of *Microcystis aeruginosa* PCC 7806 control cultures and glyphosate treatments (C1= 1.5, C2= 15, and C3=40 mg/L) grown under three diel photoperiods (h light: h dark cycles) during the light experiments. The bars are means of two (0 days), and five replicates (7 and 14 days).

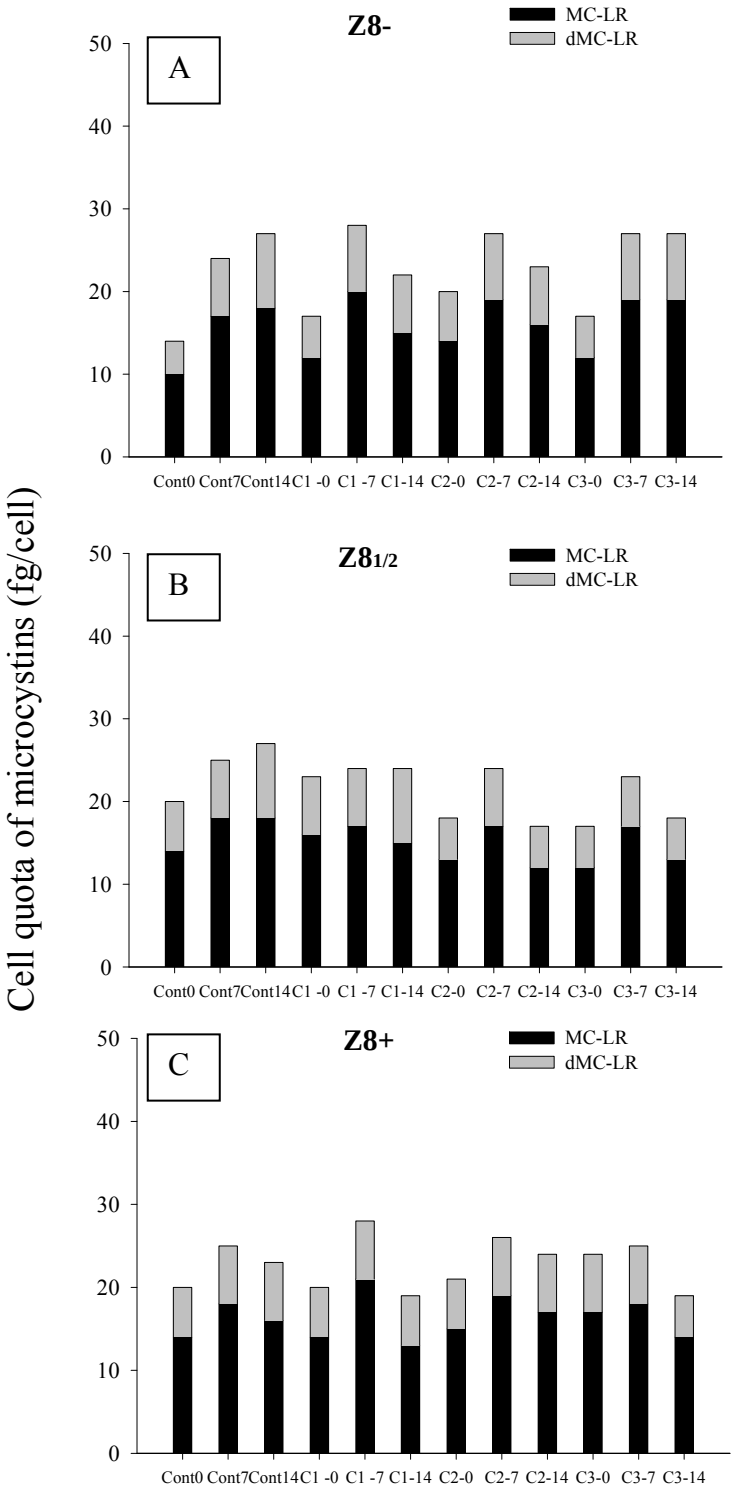

Figure 4. Time trends in average cell quotas of intracellular microcystins MC–LR and [D–Asp^3]MC–LR (fg/cell) of pre-P-starved *Microcystis aeruginosa* PCC 7806 control cultures and glyphosate treatments (C1=1.5, C2=15, and C3=40 mg/L) grown at three phosphorus concentrations (Z8: no P, Z8½ ~ 2.7 mg/L P, and Z8+ ~ 5.4 mg/L P) at a 12:12 photoperiod. The bars are means of two (0 days), and three replicates (7 and 14 days).

The ratio MC-LR to [D–Asp^3]MC–LR in the medium was around 0.9–1.0 at the beginning of the light experiments, that is, both variants were equally contributing to the extracellular pool (data not presented). Both analogs were found extracellularly at the highest glyphosate concentration after 14 days in the 12:12 in a ratio around 2.8, implying that MC–LR was the main contributor to the extracellular pool (75 %). The ratio MC-LR to [D–Asp^3]MC–LR after 14 days at a 16:8 photoperiod was around 1.5, which implies that MC–LR was the main contributor to the pool (60%).

3.5.2 Phosphorus experiments

The intracellular cell quotas of MCs in this batch were lower (20-30 fg/cell) (Fig. 4) than the quotas in the light experiments (30-40 fg/cell) (Fig 3A), probably due to the P-starvation. There were all kinds of temporal patterns for the quota that is, ascending (e.g. control at Z8- and Z8½), triangular (e.g., C1 treatment at Z8+), asymptotic (C3 treatment at Z8-), and constant (C1 at Z8½). The quotas of both MCs in the C2 (p=0.000–0.003) and C3 treatments (p=0.000–0.002) were significantly different to their controls in the Z8½ batch along the 14 days of incubation (p=0.000), and indistinguishable in the Z8- assay. In the Z8+, only the [D–Asp^3]MC–LR quota in the C1 treatments was found statistically different from the controls (p=0.033). MC–LR was again the main contributor to the microcystins pool (65–75 %), but with some minor differences per batch. The cell quotas of the microcystins were found significantly different (p=0.000) along with their interaction time × toxin during the 14 days of incubation in the Z8½ and Z8+ batches (0.000), but not during the first week of incubation for the Z8- batch (p=0.241).

The highest extracellular concentrations of microcystins (23–43 %) were again found at the beginning of the bioassays, the lowest detected in the Z8½ batch and the highest in the Z8- batch (Figs. 6A–C). The concentrations were higher than in the light experiments, probably because the samples in the P experiments were filtered after 12 hours of starting the assays and may imply continuous export of microcystins for at least one day. Less than 1 % of the total pool was found extracellularly after 7 days in the three batches, and after 14 days in the Z8- batch. While in the Z8½ batch, the control and C1 treatment presented the higher releases (12 %) with a decreasing percentage towards the C3 treatment (< 0.2 %).

The ratio of MC-LR to [D–Asp^3]MC–LR in the medium was between 1.3 and 1.5 at the beginning of the experiment. No microcystins were detected after 7 days in any of the batches of the experiment, but after 14 days in the Z8½ 1.2 in the control and 0.7 in the C2 treatment suggesting that the contribution by [D–Asp^3]MC–LR to the extracellular pool may become larger during glyphosate exposure (45–60 %).

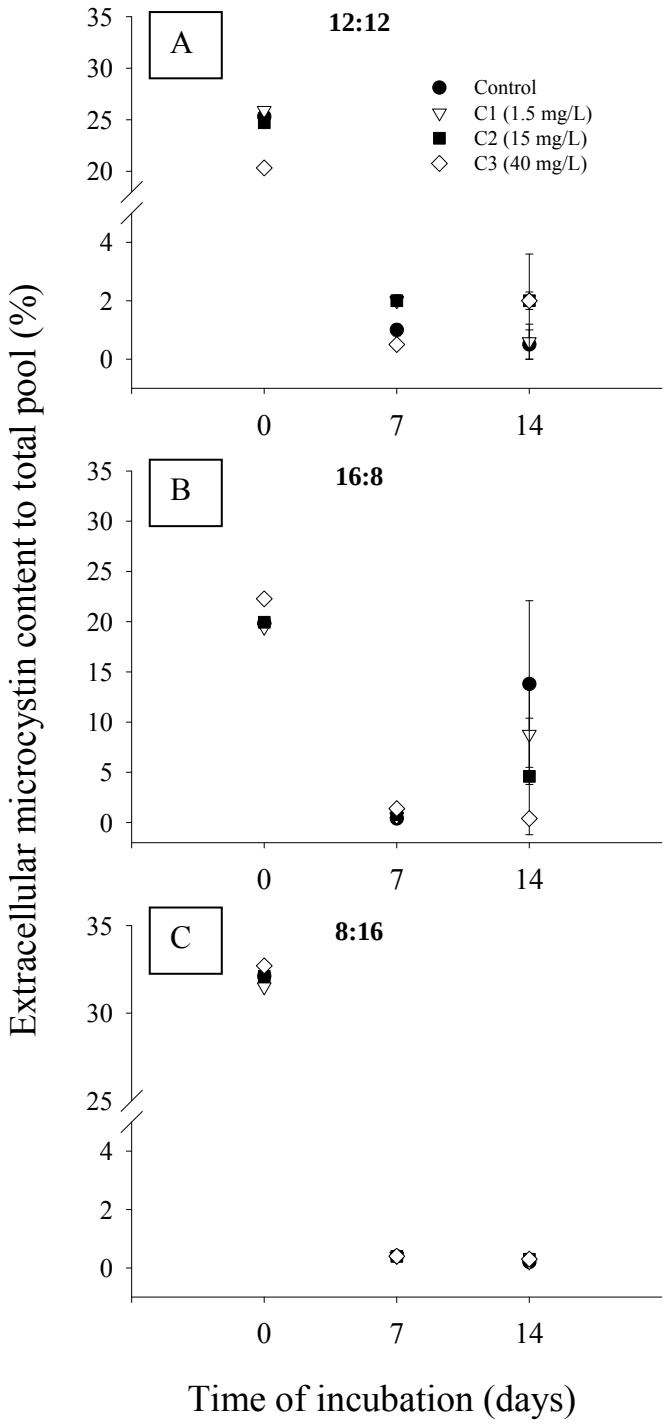

Figure 5. Extracellular microcystin volumetric content to total pool expressed as percentage in batch cultures of *Microcystis aeruginosa* PCC 7806. Control cultures and glyphosate treatments were grown under three diel photoperiods (h light: h dark cycles) during the light experiments. Symbols represent the value of one replicate (0 days), the mean of two replicates (7 and 14 days), and error bars represent the standard deviation of the means.

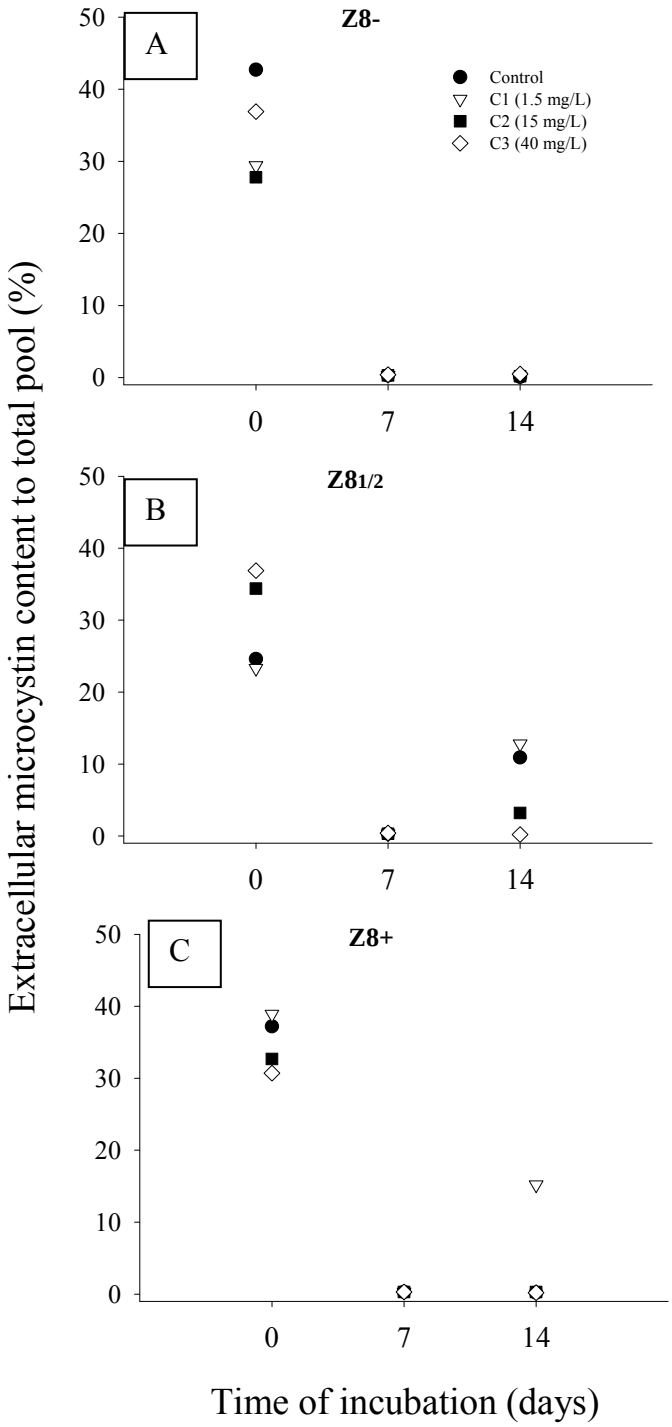

Figure 6. Extracellular microcystin volumetric content to total pool expressed as percentage in pre-P-starved *Microcystis aeruginosa* PCC 7806 control cultures and glyphosate treatments grown at three phosphorus concentrations (Z8: no P, Z8½ ~ 2.7 mg/L P, and Z8+ ~ 5.4 mg/L P) at a 12:12 photoperiod. Symbols represent the value of one replicate.

4. Discussion

This study sought to detect negative functional interactions between growth and toxin production in *M. aeruginosa* triggered by the herbicide glyphosate in cultures incubated at different light regimes and phosphorus concentrations. The trade-off was only detected when cultures were incubated under photoperiods longer than 8 h of light (12:12 and 16:8), with differential patterns per microcystin analog, time of incubation and exposure concentration. Growth was the definitively favoured trait during the first week of exposure, and less favoured over toxin production during the second week. Glyphosate could not invert the nature of the interaction between both traits along with an impressive reduction of its herbicidal action on cyanobacterial cells in the phosphorus experiments where cells were pre-P-starved, or when the cultures were incubated under the shortest photoperiod of 8 h of light. The metabolic and ecological costs involved in microcystin production are higher during the first week of incubation and minimized afterwards after comparison between cell quotas of ATP and growth rates in the wild type and the mutant cultures. Glyphosate conferred additional ecological advantages to the mutants expressed in terms of tolerance, and especially when dosed at the highest tested concentration (40 mg/L). Glyphosate, however, seem to confer benefits to the exposed cultures in the second week of incubation by either increasing toxin production or inhibiting the extracellular releases to the medium. In general, the production of MC–LR seem to be favoured over the one of [D–Asp^3]MC–LR in the first seven days of incubation, and such favoritism minimized in the last week of incubation when both productions tended to be similar or slightly higher for [D–Asp^3]MC–LR.

The relatively larger investment by the wild type into growth compared with MC production in response to the most stressful conditions in the experiment, especially in glyphosate treatments in the dim light photoperiod (8:16) and especially at the highest dose of glyphosate (Table 3), may be the expression for a basic mechanism in prokaryotes to protect survival of some cells at the sacrifice of other activities, such as toxin production. Glyphosate is known to cause cessation or retardation of growth in primary producers after blocking the aromatic amino acid synthesis and the metabolism of phenolic compounds, resulting in shortages of carbon for other essential pathways (Fischer et al. 1986, Smith and Oehme 1992).

The negative production rates calculated for [D–Asp^3]MC–LR, the rather constant relative contribution of the analog to the intracellular microcystin pool, and the low extracellular concentrations detected during the first week of incubation in the light experiments might imply that the [D–Asp^3]MC–LR pool is not stable and that the compound is synthesized, used, and replenished constantly under the given culturing conditions. This turnover of the analog seems to be triggered by glyphosate when the cultures were incubated under the 12:12 photoperiod (C2 and especially C3) and under the longest photoperiod (16:8, all treatments). Surprisingly the same phenomena was also evident in controls and treatments of the cultures incubated under the shortest photoperiod (8:16), which additionally might imply that under light scarcity situations this turnover might be genetically induced in *M. aeruginosa*. This is important in quantifyng the production costs of microcystins, and in elucidating their ecological roles (the benefits), since microcystins have always been assumed to be immobile in the cell (defenses or whatever role they have). It may also explain conflicting results from laboratory and field experiments on the effects of

environmental factors on microcystin production or accumulated concentrations. The different rates of metabolic turnover for the MC analogues is in agreement with varying turnover rates of allelopathic compounds from high rates of the polyphenol phlorotannin in tropical brown algae (Arnold and Targett 2000) to low rates for terpenoids in terpene-accumulating families (e.g., *Mentha piperita* and *Pinus contorta*) (Gershenzon (1994) and suggests that there are differential cost of production per MC analogue. MC-LR is probably the more expensive to produce and less labile than [D–Asp^3]MC–LR, since it was always the main contributor to the intracellular MCs pool, and only sacrificed under extreme situations like in the longest photoperiod under exposure to the highest glyphosate concentration.

The lower susceptibility of the wild type to glyphosate during the first week in the absence (Z8-) or in the presence of reduced P concentrations (Z8½) compared with its response at full P supply (Z8+) and in the photoperiod assays without pre-P-starvation (Table 1) may be explained by the possibility that glyphosate was utilized as a phosphate source by the cells. This is in agreement with the findings that P-starved and glyphosate exposed (EC50=9 mg/L) *Anabaena variabilis* L. increased its extracellular alkaline phosphatase activity by 167 % compared with a control and grew successfully (Ravi and Balakumar, 1998). They hypothesized that the high secretion of alkaline phosphatase into the medium cleaved the C–P bond in glyphosate and allowed *Anabaena* to utilize it as a phosphate source. Another species used in the same work, *Nostoc* did not survive in the P-starved medium and did not change the activity of the alkaline phosphatase. The controls of both strains in the P batch (especially the wild-type cells in the Z8- bioassays), exhibited impressively higher rates compared to the ones calculated under P–replete conditions (first batch 12:12) in the first 7 days of incubation which enlighten the remarkable role of internal phosphorus reserves to allow cell survival and even growth under conditions of nutrient deficiency (Cembella et al. 1984). The significantly higher ATP quotas in the wild type cells compared to the mutants in the first seven days of incubation in the batch, especially in the Z8- bioassay, take us to hypothesize that the mutant probably has a poorer or less efficient ability to mobilize the stored phosphorus (Pi) and transfer it to ATP, which is known to occur under phosphorus deficiency (Cembella et al. 1984). This loss of capacity in the mutant to mobilize internal P resources is also indicated in its susceptibility to glyphosate during the first week of incubation in the Z8- and Z8½ medium (Fig. 2). Moreover, Young et al. (2005) by immunogold labelling localized microcystins preferentially associated with the thylakoids and polyphosphate bodies-periphery in our study strain *M. aeruginosa* PCC 7806. All this might imply a potential role of microcystins into phosphorus storage dynamics.

The costs of plant chemical defenses are not always apparent, in spite of evidences that suggest that their synthesis and storage might have important metabolic costs, implying that such costs may be quite low or only evident at particular developmental stages or under specific environmental conditions (Gershenzon 1994). Several metabolic adaptations could reduce costs of chemical defenses, by playing multiple roles, such as a monoterpene acting as a defense against hervibores and pathogens and serving as an antioxidant (Gershenzon 1994). Curiously, some compounds as algal polyphenolics and plant terpenes possess multiple secondary roles or a combination of both secondary and primary roles (Gershenzon 1994, Arnold and Targett 2002) which could also apply to microcystins. Microcystins may also have an extracellular role in stress signaling, as indicated by the finding of the highest

microcystin concentrations in the medium at the beginning of all the assays, especially in the pre-P-starved cultures and the differential composition of the extracellular pool (50:50 or 60:40, MC–LR:[D–Asp^3]MC–LR) compared to the intracellular (70:30).

Growth seems to be a conservative trait in *M. aeruginosa*, which makes it the strongest endpoint to detect effects of contamination over microcystin production. However, microcystin production, and especially [D–Asp^3]MC–LR production was more responsive to glyphosate in time and at lower concentrations, that is, more sensitive, which is another desirable characteristic of ecotoxicological endpoints.

5. Acknowledgments

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6. References

Arnold, T. M., and Targett, N. M., 2000. Evidence for metabolic turnover of polyphenolics in tropical brown algae. J. Chem. Ecol. 26, 1393-1409.

Arment, A. R., and W. W. Carmichael, 1996. Evidence that microcystin is a thio-template product. J. Phycol. 32, 591-597.

Battaglin, W. A., Kolpin, D. W., Scribner, E. A., Kuivila, K. M., and Sandstrom, M. W., 2005. Glyphosate, other herbicides, and transformation products in midwestern streams, 2002. J. Am. Water Resour. Assoc. 41, 323-332.

Benjamin, R. B., and Klaine, S. J., 1995. Fitness trade-offs of *Selenastrum capricornutum* strains selected for rapid growth on copper-spiked solidified nutrient medium. Environ. Toxicol. Chem. 14, 1789-1798.

Calow, P., 1991. Physiological costs of combating chemical toxicants: ecological implications. Comp. Biochem. Physiol. 100C, 3-6.

Carmichael, W. W., 1992. Cyanobacteria secondary metabolites- the cyanotoxins. J. Appl. Bacteriol. 72, 445-459.

Chorus, I., and Bartram, J., 1999. Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. WHO, Suffolk.

Congdon, J. D., Dunham, A. E., Hopkins, W. A., Rowe, C. L., and Hinton, T. G., 2001. Resource allocation-based life histories: a conceptual basis for studies of ecological toxicology. Environ. Toxicol. Chem. 20, 1698-1703.

Dittmann, E., and Börner, T., 2005. Genetic contributions to the risk assessment of microcystin in the environment. Toxicol. Appl. Pharmacol. 203, 192-200.

Dittmann, E., Erhard, M., Kaebernick, M., Scheler, C., Neilan, B. A., Von Döhren, H., and Börner, T., 2001. Altered expression of two-light dependent genes in a microcystin-lacking mutant of *Microcystis aeruginosa* PCC 7806. Microbiology 147, 3113-3119.

Dittmann, E., Neilan, B. A., Erhard, M., Von Döhren, H., and Börner, T., 1997. Insertional mutagenesis of a peptide synthetase gene that is responsible for hepatotoxin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806. Molecular Microbiology 26, 779-787.

Donaldson, J. R., Kruger, E. L., and Lindroth, R. L., 2006. Competition- and resource-mediated trade-offs between growth and defensive chemistry in trembling aspen (*Populus tremuloides*). New Phytologist 169, 561-570.

Fischer, R. S., Berry, A., Gaines, C. G., and Jensen, R. A., 1986. Comparative action of glyphosate as a trigger of energy drain in eubacteria. J. Bacteriol. 168, 1147-1154.

Gershenzon, J., 1994. Metabolic costs of terpenoid accumulation in higher plants. J. Chem. Ecol. 20, 1281-1328.

Hesse, K., Dittmann, E., and Börner, T., 2001. Consequences of impaired microcystin production for light-dependent growth and pigmentation of *Microcystis aeruginosa* PCC 7806. FEMS Microbiol. Ecol. 37, 39-43.

Jespersen, A. M., and Christoffersen, K., 1987. Measurements of chlorophyll a from phytoplankton using ethanol as extraction solvent. Arch. Hydrobiol. 109, 445-454.

Kaebernick, M., Neilan, B. A., Börner, T., and Dittmann, E., 2000. Light and the transcriptional response of the microcystin biosynthesis gene cluster. Appl. Environ. Microbiol. 66, 3387-3392.

Kammenga, J. E., Van Koert, P. H. G., Koeman, J. H., and Bakker, J., 1997. Fitness consequences of toxic stress evaluated within the context of phenotypic plasticity. Ecol. Applic. 7, 726-734.

Kilham, P., and Hecky, R. E., 1988. Comparative ecology of marine and freshwater phytoplankton. Limnol. Oceanogr. 33, 776-795.

Lürling, M., and Van Donk, E., 2000. Grazer-induced colony formation in *Secenedesmus*: are there costs to being colonial. Oikos 88, 111-118.

Marie, D., Simon, N., and Vaulot, D., 2005. Phytoplankton cell counting by flow cytometry. In Andersen, R. A. (Eds.), Algal culturing techniques, Elsevier Inc., pp. 253-266.

Mikalsen, B., Boison, G., Skulberg, O. M., Fastner, J., Davies, W., Gabrielsen, T. M., Rudi, K., and Jakobsen, K. S., 2003. Natural variation in the microcystin synthetase operon *mcy*ABC and impact on microcystin production in *Microcystis* strains. J. Bacteriol. 185, 2774-2785.

Napolitano, M., and Shain, D. H., 2005. Quantitating adenylate nucleotides in diverse organisms. J. Biochem. Biophys. Meth. 63, 69-77.

Orr, P. T., and Jones, G. J., 1998. Relationship between microcystin production and cell division rates in nitrogen-limited *Microcystis aeruginosa* cultures. Limnol. Oceanogr. 43, 1604-1614.

Paerl, H. W., 1988. Growth and reproductive strategies of freshwater blue-green algae (cyanobacteria). In Sandgren, C. D. (Eds.), Growth and reproductive strategies of freshwater phytoplankton, Cambridge University Press, Cambridge, pp. 261-315.

Pintar, J., and Starmer, W. T., 2003. The costs and benefits of killer toxin production by the yeast *Pichia kluyveri*. Antoine Van Leeuwenhoek 83, 89-98.

Repka, S., M. Koivula, V. Harjunpä, L. Rouhiainen, and K. Sivonen, 2004. Effects of phosphate and light on growth of and bioactive peptide production by the cyanobacterium *Anabaena* strain 90 and its anabaenopeptilide mutant. Appl. Environ. Microbiol. 70, 4551-4560.

Reynolds, C., 2006. Growth and replication of phytoplankton. In Reynolds, C. S. (Eds.), The ecology of phytoplankton, Cambridge University Press, Cambridge, pp. 178-236.

Rivasseau, C., Martins, S., and Hennion, M.-C., 1998. Determination of some physicochemical parameters of microcystins (cyanobacterial toxins) and trace level analysis in environmental samples using liquid chromatography. Journal of Chromatography A 799, 155-169.

Smith, E. A., and Oehme, F. W., 1992. The biological activity of glyphosate to plants and animals: a literature review. Vet. Human Toxicol. 34, 531-542.

Steinberg, P. D., 1995. Seasonal variation in the relationship between growth rate and phlorotannin production in the kelp *Ecklonia radiata*. Oecologia 102, 169-173.

Tillet, D., Dittmann, E., Erhard, M., Von Döhren, H., Börner, T., and Neilan, B. A., 2000. Structural organization of microcystis biosynthesis in *Microcystis aeruginosa* PCC 7806: an integrated peptide-polyketide synthetase system. Chem. Biol. 7, 753-764.

Townsend, C. R., and Calow, P., 1981. Physiological ecology: an evolutionary approach to resource use. Blackwell Scientific Publications Ltd., Oxford.

Utkilen, H., and Gjølme, N., 1995. Iron-stimulated toxin production in *Microcystis aeruginosa*. Appl. Environ. Microbiol. 61, 797-800.

Vézie, C., Rapala, J., Vaitomaa, J., Seitsonen, J., and Sivonen, K., 2002. Effect of nitrogen and phosphorus on growth of toxic and nontoxic *Microcystis* strains and on intarcellular microcystin concentrations. Microb. Ecol. 43, 443-454.

Wood, A. M., Everroad, R. C., and Wingard, L. M., 2005. Measuring growth artes in microalgal cultures. In Andersen, R. A. (Eds.), Algal culturing techniques, Elsevier Inc., pp. 269-285.

Yoshida, M., Yoshida, T., Takashima, Y., Hosoda, N., and Hiroishi, S., 2007. Dynamics of microcystin-producing and non-microcystin-producing *Microcystis* populations is correlated with nitrate concentration in a Japanese lake. FEMS Microbiol. Lett. 266, 49-53.

Yoshida, T., Hairston Jr., N. G., and Ellner, S. P., 2004. Evolutionary trade-off between defence against grazing and competitive ability in a simple unicellular alga, *Chlorella vulgaris*. Proc. R. Soc. Lond. B 271, 1947-1953.

Young, F. M., Thomson, C., Metcalf, J. S., Lucocq, J. M., and Codd, G. A., 2005. Inmunogold localisation of microcystins in cryosectioned cells of *Microcystis*. J. Struct. Biol. 151, 208-214.

Zera, A. J., and Harsham, L. G., 2001. The physiology of life-history trade-offs in animals. Annu. Rev. Ecol. Syst. 32, 95-126.

Appendix A (Tables)

Table 1. Average specific growth rates (μ, day^{-1}) in batch cultures of *Microcystis aeruginosa* PCC 7806 and its mutant unable to produce microcystins PCC 7806 Δ*mcy*B. Control cultures and glyphosate treatments (C1=1.5, C2=15, and C3=40 mg/L) were incubated during 14 days. The rates were derived from 7 days logarithmic changes in cell density of three (batch IV), and five replicates (other batches), respectively. Negative values of the ratio between growth rates of the mutant and the PCC 7806 imply that rates in the wild type strain were negative. The light experiments were performed under different photoperiods h light: h darkness, and in the phosphorus experiments Z8: no P, Z8½ ~ 2.7 mg/L P, and Z8+ ~ 5.4 mg/L P.

Manipulated factor	Groups	μ PCC		μ Mutant		Ratio μ Mutant/ μ PCC	
		0–7 d	7–14 d	0–7 d	7–14 d	0–7 d	7–14 d
Light (12:12)	Control	0.045	0.141	0.100	0.141	2.2	1.0
	C1	0.059	0.136	0.114	0.115	1.9	0.8
	C2	0.044	0.135	0.114	0.140	2.6	1.0
	C3	-0.017	-0.026	0.013	0.208	-2.3	-9.0
Light (16:8)	Control	0.082	0.137	0.163	0.123	2.0	0.9
	C1	0.071	0.137	0.140	0.178	2.0	1.3
	C2	0.056	0.151	0.163	0.148	2.9	1.0
	C3	0.005	0.154	0.111	0.154	22.0	1.0
Light (8:16)	Control	0.059	0.122	0.151	0.130	2.6	1.1
	C1	0.083	0.104	0.133	0.118	1.6	1.1
	C2	0.051	0.119	0.161	0.126	3.2	1.1
	C3	0.064	0.109	0.138	0.128	2.2	1.2
Phosphorus Z8-	Control	0.086	0.087	0.132	0.100	1.5	1.2
	C1	0.071	0.122	0.152	0.100	2.1	0.8
	C2	0.089	0.106	0.134	0.133	1.5	1.2
	C3	0.055	0.109	0.144	0.117	2.6	1.1
Z8½	Control	0.072	0.088	0.166	0.095	2.3	1.1
	C1	0.099	0.076	0.171	0.098	1.7	1.3
	C2	0.084	0.117	0.168	0.097	2.0	0.8
	C3	0.060	0.127	0.162	0.131	2.7	1.0
Z8+	Control	0.104	0.085	0.174	0.069	1.7	0.8
	C1	0.072	0.100	0.130	0.068	1.8	0.7
	C2	0.092	0.104	0.175	0.087	1.9	0.8
	C3	0.085	0.112	0.165	0.091	1.9	0.8

Table 2. Average specific production rates (day^{-1}) of MC–LR and [D–Asp^3]MC–LR and its ratio in batch cultures of *Microcystis aeruginosa* PCC 7806 exposed during 14 days to three concentrations of glyphosate (C1=1.5, C2=15, and C3=40 mg/L). The rates were derived from 7 days logarithmic changes in bulk volumetric microcystin concentration (intra + extracellular, µg/L) of three (batch IV), and five replicates (other batches) respectively. The light experiments were performed under different photoperiods h light: h darkness, and in the phosphorus experiments Z8: no P, Z8½ ~ 2.7 mg/L P, and Z8+ ~ 5.4 mg/L P.

Batch/ Groups	MC–LR		[D–Asp^3]MC–LR		Ratio MC–LR/ [D–Asp^3]MC–LR	
	0–7 d	7–14 d	0–7 d	7–14 d	0–7 d	7–14 d
12:12						
Control	0.068	0.053	0.016	0.062	4.2	0.9
C1	0.053	0.070	0.011	0.067	4.8	1.0
C2	0.037	0.083	-0.006	0.088	7.2	0.9
C3	-0.042	0.043	-0.081	0.025	1.9	1.7
16:8						
Control	0.061	0.062	0.004	0.103	15.2	0.6
C1	0.047	0.072	-0.022	0.143	3.1	0.5
C2	0.031	0.122	-0.038	0.169	2.2	0.7
C3	-0.020	0.127	-0.055	0.139	2.8	0.9
8:16						
Control	0.013	0.110	-0.071	0.104	6.5	1.1
C1	0.018	0.071	-0.064	0.071	4.6	1.0
C2	0.006	0.069	-0.074	0.053	13.3	1.3
C3	0.007	0.061	-0.066	0.047	9.4	1.3
Z8$^-$						
Control	0.092	0.094	0.060	0.109	1.5	0.9
C1	0.099	0.086	0.064	0.109	1.6	0.8
C2	0.094	0.080	0.070	0.084	1.3	1.0
C3	0.052	0.115	0.017	0.110	3.1	1.0
Z8½						
Control	0.072	0.103	0.043	0.148	1.7	0.7
C1	0.081	0.070	0.047	0.141	1.7	0.5
C2	0.076	0.065	0.042	0.081	1.8	0.8
C3	0.054	0.091	0.011	0.105	4.9	0.9
Z8$^+$						
Control	0.080	0.069	0.029	0.095	2.8	0.7
C1	0.092	0.058	0.048	0.093	1.9	0.6
C2	0.073	0.089	0.039	0.098	1.9	0.9
C3	0.050	0.079	0.021	0.076	2.4	1.0

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REDUCED GRAZING RATES IN *DAPHNIA PULEX* CAUSED BY CONTAMINANTS: IMPLICATIONS FOR TROPHIC CASCADES

GÖRAN BENGTSSON,*† LARS-ANDERS HANSSON,† and KATIA MONTENEGRO†‡
†Lund University, Department of Ecology, Sölvegatan 37, SE-22362 Lund, Sweden
‡CIRA-UNAN, Aptdo. Postal 4598, Managua, Nicaragua

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Abstract—Ecotoxicological endpoints based on behavioral traits (e.g., predator avoidance, feeding, and locomotion) may be more sensitive and give more insights into patterns of sublethal toxicity than survivorship tests. In this study, the density-dependent grazing rate of *Daphnia pulex* pre-exposed to *p,p′*-dichlorodiphenyldichloroethylene (DDE) (insecticide metabolite) and glyphosate (herbicide), via water or a vector, *Scenedesmus* spp., was assayed in laboratory experiments. The phytoplankton biomass was estimated from the chlorophyll content, and the pesticide uptake and turnover pattern in *Daphnia* and *Scenedesmus* were determined from parallel experiments with a radiolabeled source. *Scenedesmus* spp. relative net growth rate was inversely and linearly related to the density of the grazer. *Daphnia pulex* exhibited significant reductions in grazing rate: 30% for those pre-exposed to *p,p′*-DDE via water and 40% for *D. pulex* pre-exposed to glyphosate via *Scenedesmus* spp. Through the process of trophic cascading, this impaired grazing allowed *Scenedesmus* spp. to grow at higher rates, 70 and 60%, respectively. The reduced grazing efficiencies were associated with the treatments that gave the highest body burden of *p,p′*-DDE (70 μg/g dry wt) and the lowest of glyphosate (13 mg/g dry wt). The pattern of results suggests a toxic effect of *p,p′*-DDE on *D. pulex* and a growth enhancement of *Scenedesmus* spp. in response to nitrogen and phosphorus in glyphosate excreted by *D. pulex*.

Keywords—Behavioral endpoint *Scenedesmus* Body burden Glyphosate Dichlorodiphenyldichloroethylene

INTRODUCTION

Applied successfully to explain differences in primary productivity among lakes with contrasting food webs, the concept of trophic cascading has moved attention toward the interactions between herbivorous zooplankton and phytoplankton and away from the complementary nutrient supply processes [1,2]. It is difficult to generalize about the conditions that are conducive to trophic cascading, from the level of piscivorous fish to phytoplankton [3]. Nonetheless, numerous studies demonstrate the positive effects of planktivorous fish on phytoplankton biomass and accumulation [4,5], and others reveal the inverse relationship of the numbers of large-bodied zooplankton with the quantity and net production rates of phytoplankton [6].

Xenobiotics, such as insecticides and metals, can affect primary production in much the same way as nutrient supply. Higher trophic levels (e.g., piscivores) are more susceptible than phytoplankton to xenobiotics, and they propagate their effects down food webs via predator performance [7]. Herbivores (e.g., zooplankton) often are more susceptible to pesticides, especially insecticides, than are phytoplankton, and median effect concentration (EC50) values for these two groups may differ by orders of magnitude [8]. Pesticide effects on zooplankton may be transmitted, both up the food webs, reducing fish growth, and down the food webs increasing primary production. The resulting phytoplankton bloom is a characteristic secondary effect of xenobiotic exposure in aquatic ecosystems [9].

Reduced grazing pressure by zooplankton on phytoplankton may follow from sublethal pesticide effects on feeding behavior (e.g., filtration, ingestion, and assimilation rates) and on locomotory behavior, such as swimming performance, coordination, predator avoidance, and prey handling [10]. Feeding behavior has been recognized as an ecotoxicological endpoint in filter feeders [11] because it has a physiological implementation closely associated with growth, metabolism, and reproduction [12]. Further, feeding behavior has a higher sensitivity than mortality. For instance, the median lethal concentration (LC50) for the insecticide fenvalerate is 0.21 μg/L in *Ceriodaphnia lacustris*, but the filtration rate decreases significantly only at 0.01 μg/L [12].

Daphnids are key species in freshwater ecosystems (e.g., as grazers on phytoplankton) and a favorite food item for many predators [13,14]. Their ability to feed efficiently is, at the same time, their success and a major disadvantage because, in so doing, they are more exposed to contamination and thus, more susceptible, which explains their frequent use in ecotoxicological tests. Xenobiotics, inorganic as well as organic, may affect food filtering and subsequent food processing (ingestion and assimilation) in *Daphnia*, causing a net reduction in rates of food attainment [15]. Filtration rates in *Daphnia* species are affected by fenvalerate [16] and endosulfan and diazinon [17]. Ingestion rates are less sensitive to toxic compounds than filtration rates [15,17], and assimilation seems to be the least sensitive process. In order for a concentration of a compound to cause significant effects on assimilation it must be close to the LC50 [16].

The present study is an effort to quantify the impact of two xenobiotics, *p,p′*-dichlorodiphenyldichloroethylene (DDE) and glyphosate, on phytoplankton growth rate due to cascading through reduced grazing rate in *Daphnia pulex*. Herein we also report on development of a bioassay for *Daphnia* species with grazing efficiency as an ecotoxicological endpoint. The

* To whom correspondence may be addressed (goran.bengtsson@ekol.lu.se).

p,p′-DDE and glyphosate represent different types of substances due to their physical–chemical properties (e.g., lipophilic vs hydrophilic), their mode and site of action, and their application targets (insecticide metabolite vs herbicide). Special attention was given to the importance of the source of contaminant exposure, because the literature is equivocal as to what extent exposure through water or food makes a contribution to the body burden of contaminants in aquatic organisms.

We compared the grazing rate of *D. pulex* on *Scenedesmus* spp. in laboratory-controlled uncontaminated conditions with those in which *D. pulex* had been pre-exposed to sublethal concentrations of *p,p′*-DDE and glyphosate through water and algae. We assumed that the growth rate of *Scenedesmus* spp. would be negatively and linearly related to the density of *D. pulex*, reflecting a constant individual grazing rate for the given set of growth conditions. Further, that the grazing rate would be reduced for *D. pulex* pre-exposed to the contaminants, and that this reduction would be greater in *D. pulex* pre-exposed to *p,p′*-DDE than to glyphosate due to differences in mode of toxic action in animal cells.

MATERIALS AND METHODS

Test species

Specimens of *Daphnia pulex* De Geer were collected in cohorts between May and October 2000 from a 50-m^2 pond in a recreational area of Lund, southern Sweden, using a plankton net (mesh size 150 μm) towed horizontally just below the water surface. The animals were kept in plastic aquaria (6 L) with Whatman glass fiber/C (Maidstone, UK) filtered copper-free tap water (pH 8.1, electrolytic conductivity 160 μS/cm, total hardness 58.4 mg/L as $CaCO_3$), which was replaced twice a week. The laboratory cultures were fed with suspensions of *Scenedesmus* spp. every second or third day, as needed, depending on the density of the cultures. Following acclimation in the aquaria for 3 to 4 d, *D. pulex* individuals, 1.3- to 1.9-mm long, were chosen for the experiments. *Scenedesmus* spp. was obtained from an in-house culture that was maintained continuously in filtered copper-free tap water, and amended with 10 ml of a commercial liquid fertilizer once a week (N 1.8%, P 1.8%, K 1.6% w/v). The same room (temperature-controlled, [20 ± 1°C] and illuminated by four 58-W fluorescent lamps, with a daily 12:12-h light:dark cycle) was used for maintenance of both the animal and algal cultures and for the experiments. The algal cultures were supported by extra lights (60-W bulb, 24 h/d) and aerated by a diaphragm air pump for 12 nonconsecutive h daily.

Chemicals

The *p,p′*-DDE (International Union of Pure and Applied Chemistry: 1,1-dichloro-2,2-bis[4-chlorophenyl]ethylene) is an impurity and the main dehydrochlorination metabolite of DDT with noncommercial use [8]. The DDT is a nonsystemic, mixed isomeric organochlorine insecticide, acting via direct contact and through ingestion [8]. The predominant constituent is *p,p′*-DDT. It is a nerve poison by affecting the sodium balance of the nerve membranes along axons, disturbing the electrical conduction [8]. Although banned for commercial use decades ago, it is used still in some developing countries and in public health emergencies. The *p,p′*-DDE is less lethal and less lipophilic but persists within organisms and soils longer than *p,p′*-DDT, resulting in a higher biomagnification potential and a worldwide presence [8,18].

Glyphosate (International Union of Pure and Applied Chemistry: *N*-[phosphonomethyl]glycine) is a broad-spectrum, nonselective, postemergence, systemic herbicide, which acts by competitive inhibition of 5-enolpyruvylshikimic acid-3-phosphate synthase, an enzyme in the shikimic acid pathway of the aromatic amino acid biosynthesis pathways, which links primary and secondary metabolism in plants and bacteria [8]. Glyphosate is an amphoteric and hydrophilic compound that undergoes degradation to aminomethylphosphonic acid (AMPA) and is not expected to bioaccumulate [8]. At the present time, it is the largest-selling crop-protection chemical product [19].

Survival assays

N-phosphonomethyl-glycine (glyphosate) (95%; Sigma Chemical, St. Louis, MO, USA), and 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene (*p,p′*-DDE) (Sigma Chemical, 99%) working solutions were prepared in water (glyphosate) and in acetone (*p,p′*-DDE) and diluted in filtered copper-free tap water to determine the highest water concentration at which *D. pulex* and *Scenedesmus* spp. would survive a 4-d exposure. Two concentrations of each contaminant (10 and 100 mg/L of glyphosate, and 1 and 10 μg/L of *p,p′*-DDE) were used after reviewing LC50 and bioconcentration factor (BCF) data of both contaminants for the test species. Five individuals of *D. pulex* in 10 ml water and a 10-ml suspension of *Scenedesmus* spp. (~100 μg/L chlorophyll) were exposed separately in a 15-ml culture tube. The test species were assayed in parallel with a control (no contaminants added). *Daphnia pulex* was assayed by recording the number of live specimens after each day of exposure. The total chlorophyll (Chl) concentration of the *Scenedesmus* spp. suspensions was quantified at 665 and 750 nm according to Marker et al. [20], using an ultraviolet-visible spectrophotometer (Beckman DU 650; Fullerton, CA, USA) equipped with a 10-mm (path length) flow cell.

Contaminant uptake and metabolism assays

Glyphosate-(glycine-2-^{14}C) sodium salt (aqueous solution) (Sigma Chemical, 7.4 mCi/mmol) and *p,p′*-DDE-ring-UL-^{14}C (toluene solution) (Sigma Chemical, 13 mCi/mmol) were used to obtain the uptake pattern and BCF. Afterward, this information was used to assess the length of the pre-exposure and of the grazing periods for the grazing experiment.

Five *D. pulex* in 10-ml water or 10-ml algal suspension (~20 μg Chl/L for glyphosate, 100 μg Chl/L for *p,p′*-DDE) were exposed in 15-ml culture tubes to a combination of glyphosate (10 mg/L) and ^{14}C-labeled glyphosate (0.03 mg/L, 1,136 nCi) or to ^{14}C-labeled *p,p′*-DDE (5 μg/L, 213 nCi). Controls, without contaminants added, were assayed in parallel with the treatments. Five replicate culture tubes were sampled daily (scintillation counted) during 4 d of exposure to the labeled compounds.

Daphnia pulex were fixed in the culture tube with Lugol's solution and transferred with a glass Pasteur pipette (carrying a small water volume) to 6-ml scintillation vials and combined with 0.2 ml of Beckman BTS-450 tissue solubilizer. The samples were digested at room temperature for at least 48 h following this; 4 ml of Beckman Ready Organic scintillation cocktail was added to each sample with subsequent stirring. One milliliter of the culture tube solution was mixed with 5 ml of Beckman Ready Safe scintillation cocktail to quantify ^{14}C activity in the water.

The algae suspension (10 ml) was filtered onto a 0.45 μm,

25 mm diameter, HAWP Millipore® (Bedford, MA, USA) filter. Each filter was digested at room temperature for at least 48 h in a 20 ml scintillation vial with one ml of Beckman BTS-450. After digestion, 0.2 ml glacial acetic acid was added to decrease the sample chemiluminescence. Fourteen ml of Beckman Ready Organic scintillation cocktail were added to each sample with subsequent stirring. Samples of the water that passed through the filter were collected, and one ml mixed with 5 ml of Beckman Ready Safe scintillation cocktail.

All samples were stored in their respective cocktails for at least 12 h before ^{14}C activity was measured in a Beckman Model LS 6500 scintillation counter, with color quench correction and error of less than 10% disintegrations per minute (dpm). The counting efficiency was obtained from a quench curve established from a set of ^{14}C unquenched standards.

Once the uptake pattern of the contaminants was established by this assay, the length of the pre-exposure and grazing periods of the grazing experiment (see below) was determined. In parallel with the grazing assay, a small-scale experiment was performed with the labeled compounds to determine their fate during all steps of the assay. Across all assays, both organisms survived both contaminants to between 90 and 100%.

Preparation of cultures for grazing experiments

The general strategy was to pre-expose grazers to uncontaminated conditions (control animals), contaminated water, and contaminated food, and then compare their performance in assays with uncontaminated water and food.

Preparation of Scenedesmus *spp. cultures*

An inoculum of *Scenedesmus* spp. from the stock culture was added to tap water to obtain approximately 2.5 L of approximately 100 μg Chl/L in each of three 5-L beakers used for this procedure per contaminant. One beaker of each trio was spiked with the contaminant (50 mg/L for glyphosate, 5 μg/L for *p,p′*-DDE) and the other two were not (Fig. 1). The trios were incubated for 7 (glyphosate) or 4 (*p,p′*-DDE) d, and stirred slowly by hand each day and repositioned with respect to the light sources. The chlorophyll concentration was measured at the beginning and end of the incubation. A small-scale phytoplankton exposure assay in 100-ml beakers (1 replicate per treatment and per contaminant) was made with the labeled compounds (glyphosate [50 mg/L] + ^{14}C-glyphosate [0.03 mg/L, 1,136 nCi] or 5 μg/L ^{14}C-*p,p′*-DDE [213 nCi]) and inoculated with *Scenedesmus* spp. at an initial density of about 100 μg Chl/L. The cultures were incubated and treated as described above for the pre-exposure experiment. The ^{14}C activity of the phytoplankton biomass and of the water was measured after filtration at the beginning and end of the exposure period.

Pre-exposure of D. pulex

Up to 225 *D. pulex* from the stock cultures were added to each of three feeding chambers that were positioned in 5-L beakers containing 2.5 L of tap water. The feeding chambers were constructed from rectangular 500-ml plastic jars, with a plankton net fixed into the bottom and two walls (mesh size 150 μm). This allowed algae to enter the chamber from the surrounding water and *D. pulex* to feed on them. For this procedure, three beakers were used per contaminant. One of the beakers was spiked with the contaminant, 50 mg/L glyphosate, or 5 μg/L *p,p′*-DDE (Fig. 1). The feeding chambers were moved to beakers with the *Scenedesmus* spp. suspensions twice a day for 1.5 h each time to allow the animals to feed. Uncontaminated *Scenedesmus* was used to feed the animals that were pre-exposed to contaminants dissolved in water, and contaminated *Scenedesmus* was used to feed those pre-exposed to the contaminants through the food. This feeding procedure started on the fifth (glyphosate) or third day (*p,p′*-DDE) of incubation and lasted for 3 d for glyphosate and 2 d for *p,p′*-DDE.

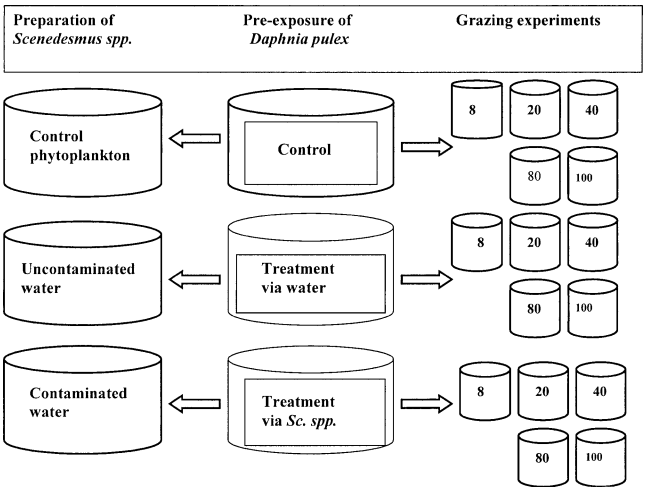

Fig. 1. Schematic representation of bioassays with *Scenedesmus* spp. and *Daphnia pulex* cultures. Preparation of *Scenedesmus*: A set of three containers were inoculated at approximately 100 μg/L of chlorophyll (Chl). These assays lasted for 2 (*p,p′*-dichlorodiphenyldichloroethylene [DDE]) or 4 (glyphosate) days. The contaminants were added to one of the containers each (contaminated water). Pre-exposure of *D. pulex*: 225 *D. pulex* were added to each of a set of three feeding chambers that were placed inside containers filled with filtered tap water. These assays lasted for 2 (*p,p′*-DDE) or 3 (glyphosate) d. The contaminants were added to one container each (treatment via water). The feeding chambers containing *D. pulex* were moved twice a day (1.5 h each time) to the container prepared with *Scenedesmus*. Grazing experiments: *D. pulex* from the pre-exposure assays were moved to containers with uncontaminated *Scenedesmus* spp. at an initial Chl concentration of about 100 μg/L. The assay was made with five densities of *D. pulex* (8, 20, 40, 80, and 100 individuals/L).

A small-scale *D. pulex* exposure assay with labeled compounds was performed to test the possibility that any changes in grazing efficiency were related to differences in body burden of contaminants in *D. pulex*. Twenty-two specimens were kept in a 50-ml glass beaker placed inside a 100-ml beaker containing 75 ml of filtered tap water. The inner beaker had three holes (two in the walls and one in the bottom) covered by plankton net (mesh size 150 μm, attached with glue) to allow algae to enter from the surrounding water. This small-scale assay was made as the pre-exposure assay described above, with three replicates used for each treatment and contaminant. The ^{14}C activity of *D. pulex* and of the assay water was measured at the beginning and end of the exposure period.

Grazing experiments

Specimens of *D. pulex* from the pre-exposure assay were added to 250 ml of filtered tap water in 1-L glass jars and inoculated with uncontaminated *Scenedesmus* spp. at approximately 100 μg Chl/L. The chlorophyll content of the water was measured before the animals were added (N_o) and at the end of the assay (N_f). The net growth rate (r) was calculated for each replicate according to $r = \ln(N_f/N_o)/\Delta t$, where Δt was the duration of the assay in days [21]. The relative change in

algal biomass was calculated as $\Delta r = r_2 - r_1$, where r_1 and r_2 are the net growth rates for *D. pulex* density 1 and 2. Five densities of *D. pulex* (8, 20, 40, 80, 100 individuals/L) were used, with three replicates per density and treatment each time the assay was made (batch). At least two batches were made per contaminant. In this way, 15 jars were used to observe grazing by *D. pulex* pre-exposed to uncontaminated water, 15 jars were used with *D. pulex* pre-exposed to glyphosate or *p,p′*-DDE dissolved in water, and 15 jars with *D. pulex* pre-exposed to *Scenedesmus* spp. contaminated with glyphosate or *p,p′*-DDE (Fig. 1). The relative net growth rates (See *Statistical analyses* section) were plotted versus the densities of *D. pulex* for each treatment. The slope of the linear regression expressed the grazing rate, and the intercept expressed the in situ algal growth rate in the absence of grazers.

The assay lasted for 4 d, and the jars were stirred daily by hand and repositioned randomly. At the end of the assay, the health condition and the number of live animals were recorded. Specimens were preserved with Lugol's solution for subsequent length measurements.

A small-scale grazing experiment was performed in parallel with the main experiment. Seven individuals of *D. pulex* were used from each of the nine replicates of the small-scale pre-exposure experiment (three replicates per treatment per contaminant). They were transferred to 100-ml beakers with 75 ml of filtered tap water inoculated with *Scenedesmus* spp. at approximately 100 μg Chl/L. The assay lasted for 4 d. The ^{14}C activity was measured in the water, *D. pulex*, and *Scenedesmus* spp. at the end of the assay.

Calculations of body burden of the contaminants in both phytoplankton and zooplankton were done based on specific activity (mCi/mmol), molecular weight of the labeled compounds (mg/mmol), and average dry weight (dry wt) of the filtered algal suspension (10 ml at different Chl concentrations) and five *D. pulex* (estimations from length). The dry weight–based bioconcentration factors (BCF_d) were calculated as (dpm in tissue/weight of tissue [g])/(dpm in water/ml of water) and measured at steady state conditions.

Statistical analyses

The algal net growth rate of the control varied from one batch to another. In order to compare results from different batches, we calculated a dimensionless relative net growth rate in the following way: Within each batch, each net growth rate (from the various treatments) was divided by the maximum net growth rate found in that batch. A confidence interval (95% probability) was used to delimit treatment data that was amenable to a statistical comparison. Normal distribution of data and homogeneity of variances were evaluated with Kolmogorov-Smirnov test and Levene's test, respectively. The observed differences between the control and contaminant treatments were evaluated by a one-way analysis of covariance, using density of *D. pulex* as the covariate, and using the interaction between treatment and density to test for the homogeneity of the slopes. All possible pairs of regression lines were compared and the significance of the regression between the relative *r* and density was assessed for each treatment and pesticide. All statistical tests were done with SPSS® for Windows 9.0.1 (Chicago, IL, USA).

RESULTS

The inverse linear relationship between the net growth rate of *Scenedesmus* spp. and the density of *D. pulex*, expressed by significant negative slopes of the regression lines (Fig. 2A,B; Density, Table 1), was taken as an estimator of grazing. In general, slopes for the control were steeper than for the treatments (Fig. 2A,B), suggesting that grazing became less efficient in *D. pulex* that were pre-exposed to the contaminants. The loss of efficiency was significant for *D. pulex* pre-exposed to *p,p′*-DDE via water (~30% compared to the control) and to glyphosate via *Scenedesmus* spp. (~40%) (Table 1, treatment·density). Hence, as a consequence of impaired grazing, the relative algal biomass increase ($\Delta r_T/\Delta r_C \cdot 100$, where T = treatment and C = control) was larger for *p,p′*-DDE (70%) than for glyphosate (60%). The *y*-intercepts, which are estimates of the net growth rate in absence of grazers, of the

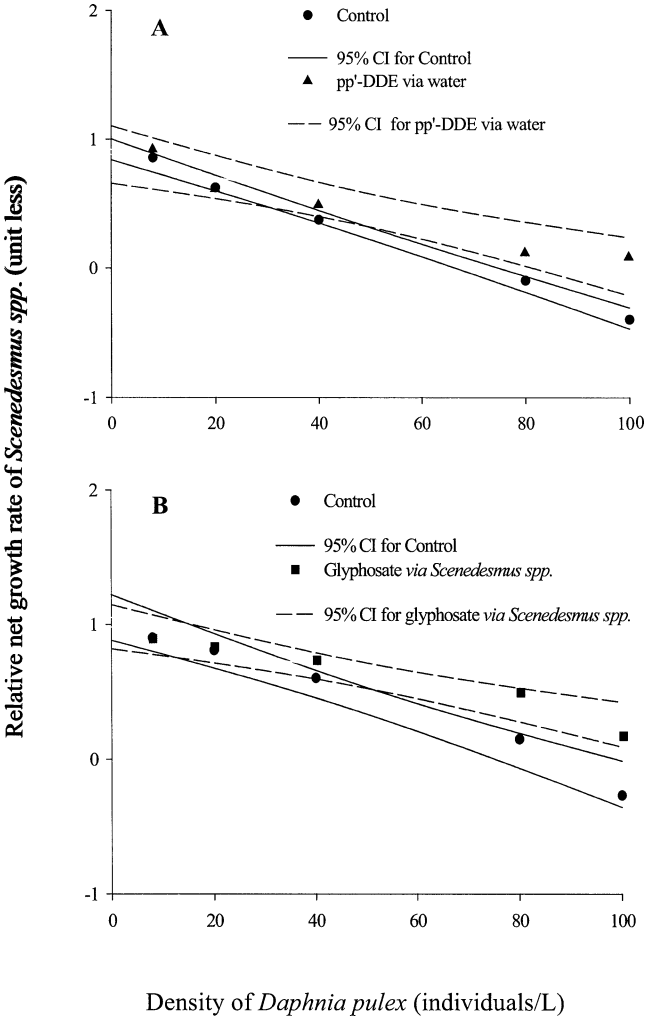

Fig. 2. Inverse linear relationship between the relative net growth rate of *Scenedesmus* spp. and density of *Daphnia pulex*. The slopes of the regression lines were estimates of the grazing efficiency (**A**) *Daphnia pulex* pre-exposed to 5 μg/L of *p,p′*- dichlorodiphenyldichloroethylene (DDE) via water ($y = -0.009x + 0.9001$, $r^2 = 0.88$) compared to *D. pulex* pre-exposed under uncontaminated conditions (control; $y = -0.013x + 0.9310$, $r^2 = 0.84$) and (**B**) *D. pulex* pre-exposed to *Scenedesmus* spp. contaminated with 50 mg/L of glyphosate (glyphosate via *Scenedesmus* spp.; $y = -0.007x + 0.9646$, $r^2 = 0.70$) compared to *D. pulex* pre-exposed under uncontaminated conditions ($y = -0.012x + 1.0478$, $r^2 = 0.97$). Means (2–4 replicates) and 95% confidence interval of the regression lines that showed significantly different slopes are given. *Daphnia pulex* pre-exposed to *p,p′*-DDE via *Scenedesmus* spp. ($y = -0.013x + 0.9836$, $r^2 = 0.69$) and to glyphosate via water ($y = -0.013x + 1.0265$, $r^2 = 0.82$), were parallel to slopes of regression lines of their respective controls (data not shown). CI = Confidence interval.

Table 1. One-way analysis of covariance of the effects of *p,p′*-dichlorodiphenyldichloroethylene (DDE) and glyphosate on the grazing efficiency of *Daphnia pulex*. The density of *D. pulex* was used as covariate and the relative net growth rate of *Scenedesmus* spp. as the dependent variable. The *y*-intercepts (treatment), the significance of the linearity of the response of the dependent on the independent variable (density), and the homogeneity of slopes (treatment·density) between relevant pairs of regression lines (control vs treatments) were tested at a significance level of 5% ($p = 0.05$)

	Control vs treatment			
	p,p′-DDE		Glyphosate	
Source of variation	Water	*Scenedesmus* spp.	Water	*Scenedesmus* spp.
Treatment	$p = 0.793$	$p = 0.812$	$p = 0.849$	$p = 0.280$
Density	$p \ll 0.050$	$p \ll 0.050$	$p \ll 0.050$	$p \ll 0.050$
Treatment·density	$p = 0.015$	$p = 0.758$	$p = 0.499$	$p = 0.001$

control and the treatments statistically were indistinguishable (Table 1, treatment). This was expected, because the algae used in the treatments had not been exposed to contaminants and were sampled from the same population as the control. The Levene's test reported a low level of significance ($p = 0.131$–0.678) for all data groups, meaning that the dependent variable was measured with the same error for control as for treatments. The error terms from the significantly different pairs of treatments were distributed normally ($p = 0.200$ in Kolmogorov-Smirnov test).

The data from the parallel small-scale experiment with ^{14}C-labeled contaminants showed that *D. pulex* took up *p,p′*-DDE and glyphosate from water as well as from *Scenedesmus* spp. (Fig. 3A,B; Table 2) and were carrying orders of magnitude higher concentrations of contaminants than the controls at the end of the grazing period. At the end of a period of pre-exposure to the contaminants via water, *D. pulex* had an average of 71 μg/g (dry wt) of *p,p′*-DDE and 50 mg/g (dry wt) of glyphosate (Table 2). The concentrations were at least 50% less when the pre-exposure was via *Scenedesmus* spp. (Table 2). *Scenedesmus* spp. took up less glyphosate (39 mg/g dry wt) and *p,p′*-DDE (30 μg/g dry wt) than *D. pulex*.

The length of the pre-exposure and grazing intervals were based on the pattern of uptake of the labeled pesticides in the separate culture tube experiments (Fig. 4A,B). The *p,p′*-DDE concentration peaked in *Scenedesmus* spp. within 12 h of exposure (Fig. 4A), corresponding to $BCF_d = 19{,}989$, and then declined within the next 36 h. The uptake of glyphosate (Fig. 4B) was slower and 3 to 4 d were required to reach a peak ($BCF_d = 2{,}000$). These observations suggested that 2 d of exposure to *p,p′*-DDE and 4 d to glyphosate were sufficient before the cultures were used to expose *D. pulex*. The *p,p′*-DDE concentration ($BCF_d = 69{,}394$) in *D. pulex* reached an equilibrium within 12 h and the glyphosate concentration ($BCF_d = 2{,}200$) within 2 to 3 d (Fig. 4A,B). Accordingly, *D. pulex* were pre-exposed to *p,p′*-DDE for 2 d and to glyphosate for 4 d before the grazing experiment started, and the grazing period was limited to 4 d.

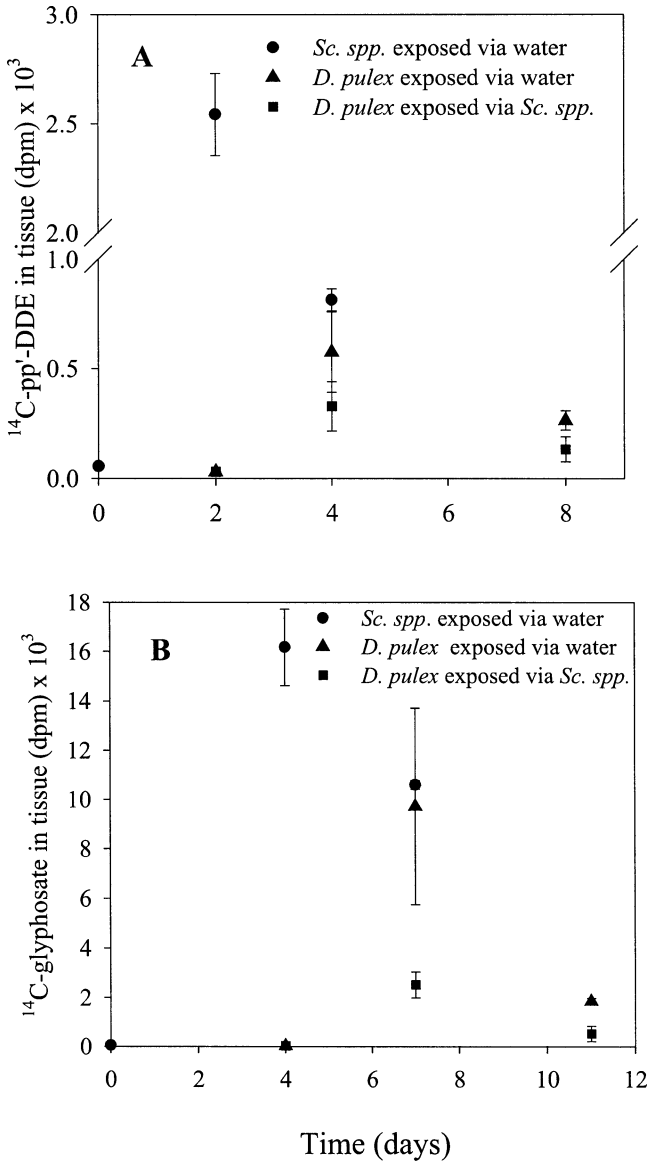

Fig. 3. ^{14}C-*p,p′*-dichlorodiphenyldichloroethylene (DDE) (**A**) and ^{14}C-glyphosate (**B**) (disintegrations per minute [dpm]) in tissue of *Scenedesmus* spp. after exposure via water and in tissue of *Daphnia pulex* after exposure via water or via *Scenedesmus spp*. were exposed for 2 (*p,p′*-DDE) or 4 (glyphosate) d. *D. pulex* were exposed (See *Pre-exposure of D. pulex* section) for another 2 (*p,p′*-DDE) or 3 (glyphosate) d to contaminated water or *Scenedesmus*. *D. pulex* were then moved to graze on uncontaminated *Scenedesmus* spp. between day 4 and day 8 (*p,p′*-DDE) and day 7 and 11 (glyphosate). Average values of three replicates and standard deviations (error bars) are given.

DISCUSSION

Although the genus *Daphnia* is recognized for its high rates of filtration of phytoplankton [6,22,23], the grazing rates realized in our assays (and in the enclosures used by Lehman and Sandgren [21]) would have only a marginal impact on the net growth rate of phytoplankton, unless the density of grazers is high (e.g., >40 individuals/L). Our results give strong support for the assumption of a linear and negative relationship between the density of *D. pulex* and the net growth rate of *Scenedesmus* spp. However, the slopes of the regression lines

Table 2. Average body burdens (three replicates) in *Daphnia pulex* and in *Scenedesmus* spp. after 2 d of exposure to ^{14}C-*p,p′*-dichlorodiphenyldichloroethylene (DDE). Calculations were made based on a specific activity of 13 mCi/mmol, molecular weight = 318 mg/mmol, the average weight of five specimens of *D. pulex* (90 μg, dry wt), and the average weight of 10 ml of *Scenedesmus* spp. suspension (950 μg, dry wt)

Test organism	dpm[a] in tissue	mCi	mmol *p,p′*-DDE	μg *p,p′*-DDE	μg/g
Daphnia pulex					
Treatment via water	573	2.6×10^{-7}	2.0×10^{-8}	6.4×10^{-3}	70.8
Treatment via *Scenedesmus* spp.	256	1.2×10^{-7}	9.0×10^{-9}	2.8×10^{-3}	31.6
Scenedesmus spp.	2,542	1.2×10^{-6}	8.9×10^{-8}	28.3×10^{-3}	29.8

[a] dpm = Disintegrations per minute.

were shallow and, for the differences between the control and treatments, significance was produced by the impact of the higher densities of *D. pulex*. By converting the phytoplankton growth rates in our study to the dimension/d, and the densities to μg/L (144–1,800) and then multiplying the resulting slopes (0.0001–0.0003/μg d) by the average density, we obtained average grazing rates of −0.089 to −0.178/d; these are more or less comparable to those from the 98 enclosures in Lehman and Sandgren [21] (average −0.053, standard deviation 0.159). The grazing rates in our controls obviously were rather high, possibly reflecting maximum biomasses that were twice as high as in most of the enclosures of Lehman and Sandgren [21], but still within one standard deviation of their mean.

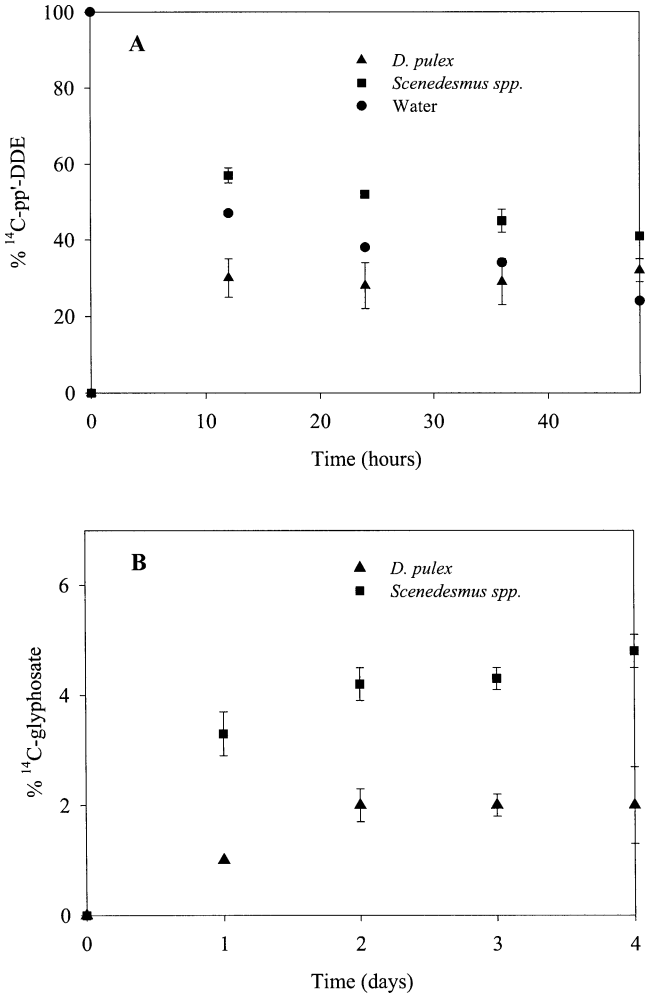

Fig. 4. Uptake of ^{14}C-*p,p′*-dichlorodiphenyldichloroethylene (DDE) (**A**) and ^{14}C-glyphosate (**B**) in *Scenedesmus* spp. and *Daphnia pulex*. The organisms were exposed in 15-ml culture tubes to the contaminants via water for 48 h (*p,p′*-DDE) and 4 d (glyphosate). The initial activity in the water was 427 ± 57 dpm for *p,p′*-DDE and 2,471 ± 41 disintegrations per minute (dpm) for ^{14}C-glyphosate. Average values and standard deviations (error bars) of five replicates are given in percentage of the initial activity in water.

The comparison here suggests that the densities of *D. pulex* used in the assays were representative of the range found in lakes and were large enough to allow detection of the impact of contaminants through grazing effects. The influence of contaminants, through grazing effects, on phytoplankton growth may be possible to observe only at relatively high grazer densities.

The reduced grazing efficiency observed in *D. pulex* pre-exposed to *p,p′*-DDE via water and pre-exposed to glyphosate via *Scenedesmus* spp. was associated with the treatments that gave the highest body burden of *p,p′*-DDE (70 μg/g dry wt) and the lowest of glyphosate (13 mg/g dry wt). Similar body burdens of *p,p′*-DDE (80 ppm dry wt) are known to cause thinning of shells in kestrel and peregrine eggs [18], and sublethal levels in the diet (10–100 μg/g) can impair reproductive behavior in guppies [24]. Insecticides, at concentrations below LC50, are known to reduce the movement of filter-feeding appendages, disrupt body coordination, and even cause immobility in zooplankton, leading to reduced filtration and/or ingestion of algal cells [11,16,17,25].

Aquatic organisms usually have much lower body burdens of glyphosate than those reported in this study [26,27]. However, glyphosate toxicity did not seem to cause the reduced grazing rate observed; we came to this conclusion because *D. pulex* pre-exposed via water had a higher body burden of glyphosate (50 mg/g dry wt) than those exposed via *Scenedesmus* spp. (13 mg/g dry wt), yet the lower level caused a greater reduction in grazing effect. A more likely explanation for this pattern of results is that *Scenedesmus* spp. growth was enhanced by excretions from *D. pulex* [28] (of glyphosate or its degradation products) that had been pre-exposed to glyphosate-contaminated *Scenedesmus* spp. During the assay, the body burden of glyphosate in *D. pulex* decreased from 13 mg/g at the beginning to 3 mg/g at the end, corresponding to a final glyphosate concentration in the assay solution of approximately 20 μg/L (2 μg/L N and 4 P). This is a concentration that has been shown to stimulate growth, photosynthesis, and chlorophyll-*a* synthesis in *Scenedesmus quadricauda* (potential bottom-up effect), probably as a result of its nitrogen or phosphorus content [29]. Further, the resulting biomass increase may slow down the filtering rates of cladocerans [30,31]. *D. pulex* pre-exposed via water also excreted glyphosate; the body burden decreased from 50 mg/g to 10, cor-

responding to approximately 75 μg/L glyphosate (6 μg/L N and 13 P) in the assay solution. The absence of a grazing effect at this higher glyphosate concentration has support in the observations by Wong [29], who noted that 200 μg/L of glyphosate had no influence on the chlorophyll-*a* content of *S. quadricauda*.

A review on freshwater cascading made by Brett and Goldman [13] showed that zooplanktivorous fish decreased zooplankton biomass by 29%, correspondingly increasing phytoplankton biomass by 176% (range from 38–711%) of the control values. Many of the studies used fish abundances that were higher than those commonly found in similar lakes. In comparison, xenobiotics such as DDT, diflubenzuron, and esfenvalerate, used at concentrations above the LC50 for zooplankton, decreased zooplankton populations by 70% (range 50–85%) with an increase in phytoplankton biomass of 230% (range 210–260%) [9,32,33]. The trophic level ratios of biomass effects, 6 (176/29) for zooplanktivorous fish and 3 (230/70) for contaminants, suggest that contaminants have at least half the impact, compared to zooplanktivorous fish, in causing cascading effects. Variations in cascading effects, to some extent, reflect variations in biological characteristics of the dominating zooplankton (e.g., large vs small species and individuals) or phytoplankton (e.g., edible green algae vs colonial cyanobacteria) [13,14]. For instance, the genus *Daphnia* has a larger impact on phytoplankton biomass than many other zooplankton genera (e.g., rotifers) [13,14].

Grazing efficiency in *D. pulex* was more sensitive (15× for glyphosate and 3× for *p,p′*-DDE) as an ecotoxicological endpoint than the 48-h LC50 for glyphosate (780 mg/L, [34]), and our estimated 48-h LC50 for *p,p′*-DDE (13.5 μg/L) (lacking data on *p,p′*-DDE LC50 for *Daphnia* spp., we estimated it from linear correlations for *p,p′*-DDT [$k_1 = 15$] and *p,p′*-DDD [$k_2 = 7.6$] between LC50s for fish and *Daphnia* spp. Once k_1 and k_2 were known, we calculated a slope k_3 [$k_1 + k_2/2$] for *p,p′*-DDE. The average LC50 for *D. pulex* was extrapolated from k_3 using averaged reported *p,p′*-DDE LC50 for fish. The calculated LC50 was compared and found to agree with the observed survival of *D. pulex* at different concentrations of *p,p′*-DDE in the assays). Median lethal concentration is a logical first, fast, and cheap endpoint for acute toxicity assessment and can be used to rank toxicity of substances whose mechanisms of action may be quite different [35,36]. Compared to LC50s, behavioral changes may be more sensitive and may anticipate reductions of survival, growth, and reproduction; further, they become apparent at shorter exposure durations and at lower contaminant concentrations [16,17,37]. For example, the increased swimming activity in *D. pulex* at sublethal concentrations of the insecticide carbaryl (one-fifth of its 48-h LC50) seems to increase the vulnerability of specimens to predation and reduce their body size by allocating energy away from growth [37]. More generally, for filter feeders there are two consequences of a decreased grazing efficiency. First, the current produced by movement of the thoracic appendages, in addition to feeding, subserves respiration, and it is essential that filtration continues even if food is not going to be collected. Second, there is a threshold concentration of food that must be ingested for an individual to fulfill its basic metabolic needs [12].

The BCF_ds for *p,p′*-DDE in this study were similar to those reported by Verschueren [8] for different organisms, and this is most likely attributed to the high K_{ow} of *p,p′*-DDE [38]. The glyphosate BCF_ds that we observed were lower than for *p,p′*-DDE, but were higher than expected from its low K_{ow}. However, higher-than-expected BCF_ds are known for other substances with negative log K_{ow}, such as methanol, ethyl acetate, and urea [39]. It is possible that these substances are taken up rapidly and then metabolized. We also observed a biodilution of both contaminants, that is, a reduction in contaminant concentration per unit weight during uptake from *Scenedesmus* spp. (Fig. 4A). This phenomenon has been reported by some authors [40,41] and mainly is attributed to high growth rates and slow partitioning of compounds between water and phytoplankton.

The bioassays demonstrated that the insecticide metabolite, *p,p′*-DDE, at concentrations above ambient levels, increased the primary producer biomass through cascading by releasing the top-down control exerted by zooplankton. Similarly, the herbicide glyphosate increased phytoplankton biomass, probably by a combination of cascading and bottom-up control. The mixed effects of glyphosate on the bioassay system call for a more detailed evaluation of its mode of action on trophic interactions of varying complexity. Though grazing assays like the one described here are laborious and critically dependent on assay conditions, they help to elucidate secondary effects of contaminants on community processes (e.g., biological interactions) that would escape detection by other ecotoxicological endpoints.

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REFERENCES

1. Paine RT. 1980. Food webs: Linkage, interaction strength, and community infrastructure. The third Tansley lecture. *J Anim Ecol* 49:667–685.
2. Hansson LA. 1992. The role of food chain composition and nutrient availability in shaping algal biomass development. *Ecology* 73:241–247.
3. Persson L. 1999. Trophic cascades: Abiding heterogeneity and the trophic level concept at the end of the road. *Oikos* 85:385–397.
4. Mills EL, Schiavone A. 1982. Evaluation of fish communities through assessment of zooplankton populations and measures of lake productivities. *N Am J Fish Manag* 2:14–27.
5. Persson L, Diehl S, Johansson L, Andersson G, Hamrin SF. 1992. Trophic interactions in temperate lake ecosystems: A test of food chain theory. *Am Nat* 140:59–84.
6. Jeppesen E, Søndergaard M, Jensen JP, Mortensen E, Hansen AM, Jørgensen T. 1998. Cascading trophic interactions from fish to bacteria and nutrients after reduced sewage loading: An 18-year study of a shallow hypertrophic lake. *Ecosystems* 1:250–267.
7. Fleeger JW, Carman KR, Nisbet RM. 2003. Indirect effects of contaminants in aquatic ecosystems. *Sci Total Environ* 317:207–233.
8. Verschueren K. 1983. *The Handbook of Environmental Data on Organic Chemicals*, 2nd ed. Van Nostrand Reinhold, New York, NY, USA.
9. Hurlbert SH. 1975. Secondary effects of pesticides on aquatic ecosystems. *Residue Rev* 57:81–148.
10. Ferrando MD, Janssen CR, Andreu E, Persoone G. 1993. Ecotoxicological studies with the freshwater rotifer *Brachionus calyciflorus*. *Ecotoxicol Environ Saf* 26:1–9.
11. Fernández-Casalderrey A, Ferrando MD, Andreu-Moliner E. 1992. Filtration and ingestion rates of *Brachionus calyciflorus* after exposure to endosulfan and diazinon. *Comp Biochem Physiol* 103:357–361.

12. Day KE, Kaushik NK, Solomon KR. 1987. Impact of fenvalerate on enclosed freshwater planktonic communities and on in situ rates of filtration of zooplankton. *Can J Fish Aquat Sci* 44:1714–1727.
13. Brett MT, Goldman CR. 1996. A meta-analysis of the freshwater trophic cascade. *Proc Natl Acad Sci USA* 93:7723–7726.
14. Scheffer M. 1998. Trophic cascades. In Usher MB, DeAngelis DL, Manly B FJ, eds, *Ecology of Shallow Lakes*, Vol 22—Population and Community Biology Series. Chapman & Hall, London, UK, pp 122–146.
15. Allen Y, Calow P, Baird DJ. 1995. A mechanistic model of contaminant-induced feeding inhibition in *Daphnia magna*. *Environ Toxicol Chem* 14:1625–1630.
16. Day K, Kaushik NK. 1987. Short-term exposure of zooplankton to the synthetic pyrethroid, fenvalerate, and its effects on rates of filtration and assimilation of the alga, *Chlamydomonas reinhardii*. *Arch Environ Contam Toxicol* 16:423–431.
17. Fernández-Casalderrey A, Ferrando MD, Andreu-Moliner E. 1994. Effect of sublethal concentrations of pesticides on the feeding behaviour of *Daphnia magna*. *Ecotoxicol Environ Saf* 27: 82–89.
18. Walker CH, Hopkin SP, Sibly RM, Peakall DB. 1996. The fate of organic pollutants in individuals and in ecosystems. In *Principles of Ecotoxicology*. Taylor & Francis, London, UK, pp 67–102.
19. Woodburn AT. 2000. Glyphosate: Production, pricing, and use worldwide. *Pest Manag Sci* 56:309–312.
20. Marker A, Nusch E, Rai H, Riemann B. 1980. The measurement of photosynthetic pigments in freshwaters and standardization of methods: Conclusions and recommendations. *Archiv für Hydrobiologie Beihefte Ergebnisse der Limnologie* 14:91–106.
21. Lehman JT, Sandgren CD. 1985. Species-specific rates of growth and grazing loss among freshwater algae. *Limnol Oceanogr* 30: 34–46.
22. Brooks JL, Dodson SI. 1965. Predation, body size, and composition of plankton. *Science* 150:28–35.
23. Pace ML. 1984. Zooplankton community structure, but not biomass, influences the phosphorus–chlorophyll-*a* relationship. *Can J Fish Aquat Sci* 41:1089–1096.
24. Bayley M, Junge M, Baatrup E. 2002. Exposure of juvenile guppies to three antiandrogens causes demasculinization and a reduced sperm count in adult males. *Aquat Toxicol* 56:227–239.
25. Crosby DG, Tucker RK. 1971. Accumulation of DDT by *Daphnia magna*. *Environ Sci Technol* 5:714–716.
26. Folmar LC, Sanders HO, Julin AM. 1979. Toxicity of the herbicide glyphosate and several of its formulations to fish and aquatic invertebrates. *Arch Environ Contam Toxicol* 8:269–278.
27. Wang Y-S, Jaw C-G, Chen Y-L. 1994. Accumulation of 2,4-D and glyphosate in fish and water hyacinth. *Water Air Soil Pollut* 74:397–403.
28. Gulati RD, Perez Martinez C, Siewertsen K. 1995. Zooplankton as a compound mineralizing and synthesizing system: Phosphorus excretion. *Hydrobiologia* 315:25–37.
29. Wong PK. 2000. Effects of 2,4-D, glyphosate and paraquat on growth, photosynthesis, and chlorophyll-*a* synthesis of *Scenedesmus quadricauda* Berb 614. *Chemosphere* 41:177–182.
30. Berman MS, Richman S. 1974. Feeding behaviour of *Daphnia pulex* from Lake Winnebago, Wisconsin, USA. *Limnol Oceanogr* 19:105–109.
31. Lampert W. 1986. Response of the respiratory rate of *Daphnia magna* to changing food conditions. *Oecologia* 70:495–501.
32. Webber EC, Deutsch WG, Bayne DR, Seesock WC. 1992. Ecosystem-level testing of a synthetic pyrethroid insecticide in aquatic mesocosms. *Environ Toxicol Chem* 11:87–105.
33. Boyle TP, Fairchild JF, Robinson-Wilson EF, Haverland PS, Lebo JA. 1996. Ecological restructuring in experimental aquatic mesocosms due to the application of diflubenzuron. *Environ Toxicol Chem* 15:1806–1814.
34. Tomlin C. 1997. *The Pesticide Manual*, 11th ed. British Crop Protection Council, Farnhman, Surrey, UK.
35. Rand GM, Wells PG, McCarty LS. 1985. Introduction to aquatic toxicology. In Rand GM, Petrocelly SR, eds, *Fundamentals of Aquatic Toxicology: Methods and Applications*. Hemisphere, Washington, DC, USA, pp 3–59.
36. Giesy JP, Graney RL. 1989. Recent developments in and intercomparisons of acute and chronic bioassays and bioindicators. *Hydrobiologia* 188/189:21–60.
37. Dodson SI, Hanazato T, Gorski RR. 1995. Behavioural responses of *Daphnia pulex* exposed to carbaryl and Chaoborus kairomone. *Environ Toxicol Chem* 14:43–50.
38. Geyer HJ, Scheunert I, Brüggemann R, Steinberg C, Korte F, Kettrup A. 1991. QSAR for chemical bioconcentration in *Daphnia*, algae, and mussels. *Sci Total Environ* 109/110:387–394.
39. Geyer H, Politzki G, Freitag D. 1984. Prediction of ecotoxicological behaviour of chemicals: Relationship between *n*-octanol/water partition coefficient and bioaccumulation or organic chemicals by alga *Chlorella*. *Chemosphere* 13:269–284.
40. Biddinger GR, Gloss SP. 1984. The importance of trophic transfer in the bioaccumulation of chemical contaminants in aquatic ecosystems. *Residue Rev* 91:103–137.
41. Swackhamer DL, Skoglund RS. 1993. Bioaccumulation of PCBs by algae: Kinetics versus equilibrium. *Environ Toxicol Chem* 12: 831–838.

Diversity–productivity relationships in pesticide-exposed Chlorophyta communities

Katia Montenegro[a,b], and Göran Bengtsson[a]

[a]Department of Ecology, Lund University, Sölvegatan 37, SE-22362 Lund, Sweden
[b]CIRA-UNAN, Aptdo. Postal 4598, Managua, Nicaragua

Abstract

The exploration of the relationships between ecosystem processes and diversity has venerable roots in ecological research and may be used to understand the dynamics in responses to contaminants. We varied algal diversity (i.e., the number of species) and the concentration of the herbicide glyphosate and the organophosphate insecticide fenthion to test for effects of combinations of them on community productivity. Communities with different number of Chlorophyta species (2, 4, 8 and 16) but the same total initial biomass were exposed to three concentration levels of the herbicide and of the insecticide during 7 days in laboratory bioassays. Productivity increased in all communities, especially in the exposed to glyphosate in a positive dose–dependent fashion probably explained by its potential use as a nutrient source, while the fenthion assay had no general pattern for the ranking of productivities among control and treatments. The most replicated trajectory of the relationship between diversity and productivity for our communities was unimodal (concave) regardless of species composition, and hence composed by an ascending part that peaked at a diversity of 4 spp. with potential to be between 4 and 7 spp., and a descending part that usually ended at the highest diversity (16 spp.), but in some instances either saturated or saturated and continued decreasing. Pesticides, in general, did not greatly change the trajectory of the relationship but its amplitude, which implies an impact on the magnitude of the effect of diversity on productivity. For example, the herbicide glyphosate produced a sharpening in the relationship given that acted as a nutrient source and hence subsidized productivity at almost all assayed concentrations, and sometimes acted as a provoker (at 1 mg L^{-1}) or as a buffer (at 50 mg L^{-1}) for competition. Fenthion only impacted the effect of diversity on productivity at concentrations between 100 and 1000 μg L^{-1}. However, in two batches we observed idiosyncratic convex relationships with different amplitudes, after the replacement of a single species (*Cosmarium botrytis*) in both batches probably mediated by competitive effects of bacteria associated to this alga, and after hyperdominance by *Chlorella fusca*. These two hidden treatments highlighted the potential important role of algae-bacteria associations and dominance on the magnitude and complexity of the community response to changing diversity. Niche complementarities, competition, and co-existence (redundancy) might provide mechanistical explanations to the observed hybrid patterns of positive, negative and asymptotic parts of the relationship, and explain the nature and strength of the operative species interactions. Species composition, on the other hand, did make an impact in the level of productivity reached in the communities, hence affecting the amplitude of the relationship diversity–productivity but not its trajectory. In general, our results suggest that declining and augmenting diversity within a community may result in local changes in productivity that might be enhanced or buffered by pesticides.

Introduction

The characterization of species assemblages has a long history in environmental assessment and has helped to gain many insights about the impact of, for example, nutrient enrichment and xenobiotics, on the intermediate complex hierarchical level represented by communities (Schindler, 1987; Ford, 1989; Clements and Newman, 2002; Stevenson and Smol, 2003). Usually, two kinds of community attributes are monitored. One is structural attributes, such as taxonomic composition, diversity, and similarity of species composition (Sheehan, 1984; Stevenson and Smol, 2003), which has received most attention (Pratt and Cairns, 1996; Cairns et al., 2003). The other is functional attributes, such as net primary productivity, transformation rates (e.g., nutrient cycling), and recovery rates after stress (i.e., resilience) (Sheehan, 1984; Levine, 1989; Cairns et al., 2003). There is an increasing appreciation that functions of communities may contain important descriptors of effects of contaminants, especially after they are described to the general public as services (Pratt and Cairns, 1996; Cairns et al., 2003).

The idea to test the relationship between diversity and functioning has venerable roots in ecology (Darwinian) and has inspired research for more than 150 years (Loreau et al., 2001; Naeem et al., 2004; Hector and Bagchi, 2007). The first approach to the relationship attempted to explain large-scale (between sites) variation in diversity by factors such as productivity, climate or disturbance regime (Bengtsson et al., 2004). The second approach, the most recent one (1990's), has turned the diversity-productivity question around, and productivity is the response (dependent variable) rather than the independent variable (Bengtsson et al., 2004). It examines if a variation in diversity at single sites affects productivity. However, the motivation and intent of early research with this second approach were centred on agro-ecological efforts to improve yield through intercropping (Trenbath, 1974; Vandermeer, 1989; Naeem et al., 2004). The recent interest in the topic is due to the realization that sustained loss of biodiversity might affect ecosystem functioning and services to humans (Hector and Bagchi, 2007).

The central question about the relationship between biodiversity and ecosystems functioning is how the shape of the trajectory might look like for a particular function for a given variation in biodiversity, for example, due to local extinction (loss) or invasion (augmentation) (Naeem et al., 2004). Over 50 different hypotheses have been put forward to associate ecosystem consequences to biodiversity changes, partly overlapping and not mutually exclusive (Giller and O'Donovan, 2002; Naeem et al., 2004). Three core ideas can be identified from the hypotheses: *i*) species are primarily singular (i.e., each species makes unique contributions), *ii*) species are primarily redundant (i.e., not all species matter), and *iii*) species impacts are context–dependent and therefore idiosyncratic (i.e., species identities rather than diversity matters) (Naeem et al., 2004). Initial explanations for the effects of plant species diversity on plant production were based on plant resource use models and focused on competition for resources and niche complementarity as the main mechanisms explaining their relationship (Tilman et al., 1997; Loreau, 1998). The modern approach treats these effects as the result of multiple causes that include sampling (selection effect) in the sense that more diverse mixtures have a higher probability of containing species with extreme traits which could become

dominant and drive ecosystem functioning, facilitation, and perhaps other causes (Naeem et al., 2004).

The relationship between structural and functional properties of a community is a classical challenge in basic disturbance ecology that has been undertaken by ecotoxicology to understand the dynamics in responses to contaminants (Sheehan, 1984; Levine, 1989; Pratt and Cairns, 1996; Clements and Newman, 2002). Predictions and empirical evidence suggest that structure (e.g., species richness) promptly responds to a number of stressors with a reduction or a shift in species assemblages (Odum, 1985; Schindler, 1987). In spite of the usually strong relationship between species richness and function, some functional changes are difficult to detect and unpredictable due to high variability and complexity (e.g., indirect effects) compared to changes in structure (Schindler, 1987; Levine, 1989; Pratt and Cairns, 1996). It is generally expected that structural attributes (diversity) would be more affected by contaminants than processes due to the so called functional redundancy (Schindler, 1987; Levine, 1989; Pratt and Cairns, 1996), which means that different species perform the same function so that changes in species diversity does not affect functioning (Loreau, 2004).

Generalizations about the response of primary production processes to pesticides are nearly impossible, because productivity is either stimulated or depressed depending on the type of pesticide, its concentration, and a number of other factors (Sheehan, 1984). For example, the versatile herbicide glyphosate has a broader utility as a growth regulator (Baylis, 2000) and is known to produce stimulatory effects in primary producers when dosed at low concentrations (Wong, 2000; Cedergreen et al., 2007; Velini et al., 2008). Fenthion, a hydrophobic organophosphate insecticide, which is acutely toxic to insects at µg L^{-1} levels (Yeh and Chen, 2006), is expected to affect algae only at high concentrations (> 1 mg L^{-1}) (USEPA, 1998; Yeh and Chen, 2006).

The objective of our study was to examine the impact of the herbicide glyphosate and the insecticide fenthion on the diversity–productivity relationship of species assemblages of the algal class Chlorophyceae. To accomplish this objective, we varied the number of species and the concentration of pesticides in experiments in which the impact on community productivity was assayed. To our knowledge, this is the first attempt to clarify the impact of pesticides on this relationship on a bivariate plane. As working hypotheses, we expected *i*) a positive asymptotic relationship between the number of species and community productivity based on the paradigm system "the mesic grasslands" (Tilman et al., 1997; Bengtsson et al., 2004), *ii*) that productivity would be stimulated by exposure to a low concentration of glyphosate, and reduced by higher concentrations affecting susceptible species, and *iii*) that exposure to fenthion at lower concentrations would not affect productivity, but higher concentrations.

Materials and methods:

Test algae

The 17 freshwater green algal strains (Appendix, Table 1) used in the experiments were obtained from the culture collection of algae at the University of Texas (http://www.bio.utexas.edu/research/utex/) and belonged to the class

Chlorophyceae. Five strains were not axenic, for example, the ones having the prefix B in their strain designation number. The stock cultures were grown in batches in filter-sterilised (0.22 μm, 47 mm Ø, GSWP Millipore filter) culture medium (USEPA, 1993) at 22 ± 2 ºC in 1 L Erlenmeyer flasks under continuous cool-white fluorescent light (2 lamps × 40 W) of 75 μE m^{-2} s^{-1} intensity and continuous aeration by diaphragm air pumps (Elite 802, China). Originally, we planned to use 29 species, but since not all of them could grow satisfactorily (contaminated or unhealthy) in the USEPA culture medium (USEPA, 1993), we finally selected the 17 species listed in Table 1. The exponential phase of growth of the cultures was estimated from their chlorophyll content. Once it was reached, the culture was immediately stored at 4 ºC to allow sedimentation and preservation until all the cultures were ready for an experimental batch (each time each experiment was made). The communities were composed from 5 to 7 days old concentrated cultures of similar chlorophyll content. Purity and good condition of each culture was verified before each experimental batch by observation of integrity of cell walls and chloroplasts on glass slides at 40× using a Leitz Laborlux D (Germany) compound microscope. The total chlorophyll content (Chl) was analyzed according to (Jespersen and Christoffersen, 1987), and it was quantified in an ultraviolet-visible (UV-Vis) spectrophotometer (PerkinElmer Lambda 35, Shelton, CT, USA) equipped with a 10-mm path length flow cell with spectral bandwidth of 0.5 nm. In the majority of the experiments, another UV-Vis spectrophotometer (Varian DMS 80, Australia) was used until we realized that the equipment was underestimating the chorophyll content by a factor of four.

Chemicals

Glyphosate (IUPAC name: N-(phosphonomethyl)glycine) was purchased from Sigma (P5671, 95%). It is a non-selective, systemic herbicide, which inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSP), an enzyme of the aromatic amino acid biosynthesis pathways, preventing the synthesis of proteins (Tomlin, 1997)(Tomlin 1997). Many physicochemical and physiological processes in primary producers are affected by glyphosate, e.g., photosynthesis and chlorophyll synthesis (Baylis, 2000). A stock solution (5000 mg L^{-1}) was prepared in deionised water and aliquots added to the experimental water (see below) at the onset of the experiments at nominal concentrations of 1 (Gly I), 10 (Gly II) and 50 (Gly III) mg L^{-1}. The three levels used for the experiments were selected based on doses reported to be close to the EEC (Expected Environmental Concentration ≈1.5 mg L^{-1}) (Battaglin et al., 2005) for aquatic exposure, No Observed Effect Concentration (NOEC=11 mg L^{-1}) and the effective concentration required to reduce the endpoint of interest by 50 % (EC50=50 mg L^{-1}) in the standard algal test species *Pseudokirchneriella subcapitata* formerly known as *Selenastrum capricornutum* Printz (USEPA, 2000).

Fenthion (IUPAC name: O,O-dimethyl-O-[4-(methylthio)-m-tolyl] phosphorothioate) was purchased from Supelco (PS-655, 98.5%). It is an organophosphate insecticide with contact, stomach and respiratory action, used to control insects in public health situations and in houses (especially as mosquito adulticide), and in the control of animal ectoparasites (Tilman et al., 1997). A stock solution (2300 mg/L) was prepared in methanol and aliquots added to the experimental water at nominal concentrations of 10 (Fen I), 100 (Fen II) and 1000 (Fen III) μg L^{-1}. The mixture was homogenized before each experiment by overnight magnetic stirring. The three levels used for the experiments were selected based on

doses reported to be close to the EEC (0.1–15 μg L^{-1}), NOEC of the most sensitive algal species (200 μg L^{-1}), and EC50 of the most tolerant algal species (1100 μg L^{-1}) (USEPA, 1998). The mixture was homogenized before each experiment by overnight magnetic stirring.

The experimental water came from the moderately polluted well La Mora in the Chinandega province, Nicaragua. It was passed through 47 mm Ø Whatman GF/C glass microfibre filters (Maidstone, England) and amended with $NaHCO_3$ (300 mg L^{-1}) as a carbon source. The pH was adjusted to ~ 7 with 0.1 N HCl to resemble the original pH of the water. The electrolytic conductivity was 670 μS cm^{-1}, alkalinity and total hardness were 250 and 140 mg L^{-1} respectively, and the nutrient content was 3 mg L^{-1} NO_3–N, and 80 μg L^{-1} PO_4–P.

Experimental design

Communities (assemblages) with different numbers of algal species, selected along a $\log_2$ species numbers gradient (2, 4, 8 and 16) but with the same initial total chlorophyll content (ca. 200 μg Chl L^{-1}), were exposed to three pesticide concentration levels (I, II, and III) during 14 days (Appendix, Table 2). Control communities were assembled without pesticides added, but for the fenthion bioassays, they contained a small amount of methanol. In general, four replicates were made for each pesticide concentration and species mixture (64 samples per batch), except for the glyphosate batches II and III, where only two replicates were prepared (32 samples per batch). The cultures were kept in 250 ml Erlenmeyer flasks capped with parafilm at 20 °C ± 2 °C, a light cycle of 14:10 (irradiance ~ 30 μE m^{-2} s^{-1}), and with orbital agitation at 100 rpm (Lab-line Instruments Inc., USA). The flasks were stirred by hand once a day and randomly repositioned according to the light supply. Samples for chlorophyll and phytoplankton counting were taken at days 0, 7 and 14.

The communities with fewer than 16 species were composed by sampling from the community with the next larger number of species (Fig. 1). To assemble the 8 species communities in the batches I, II of glyphosate and the first of fenthion, eight out of the ten genuses available were selected. Among genus with more than one species, a random sampling was made within the genus. Two characteristics were considered for assembling communities with 2 and 4 species (i.e., Glyphosate I, II): genus representativeness and expected glyphosate tolerance/susceptibility (from few reports available) (Appendix, Table 2). For example, to assemble the 4 species communities we selected 2 susceptible species (susceptible to intermediate) and 2 tolerant species (intermediate to tolerant), which belonged to a different genus. In general, intermediate susceptibility was assumed when no information about susceptibility or tolerance of a species was available. In the case of fenthion, fewer observations on tolerance/susceptibility were available for phytoplankton, and the same species as in the glyphosate batches were selected for the assemblages. The communities in the second batch of fenthion and the third of glyphosate were composed from the species that were not used in the composites of 2, 4 and 8 species of the first batch of fenthion and the second of glyphosate and randomly assembled. However, the species used in the 2 species assemblages of the second batch of glyphosate were different to the ones used in the other two subgroups of two species assemblages (Appendix, Table 2). The first glyphosate batch distinguished itself by the presence of *Chlamydomonas reinhardtii* (CIRA 6) that was replaced by

Cosmarium botrytis (CIRA 12) in the subsequent batches (Tables 1 and 2) after heavy contamination of the former stock culture. The glyphosate batches II and III were the only ones performed at the same time, and shared their 16 species assemblages.

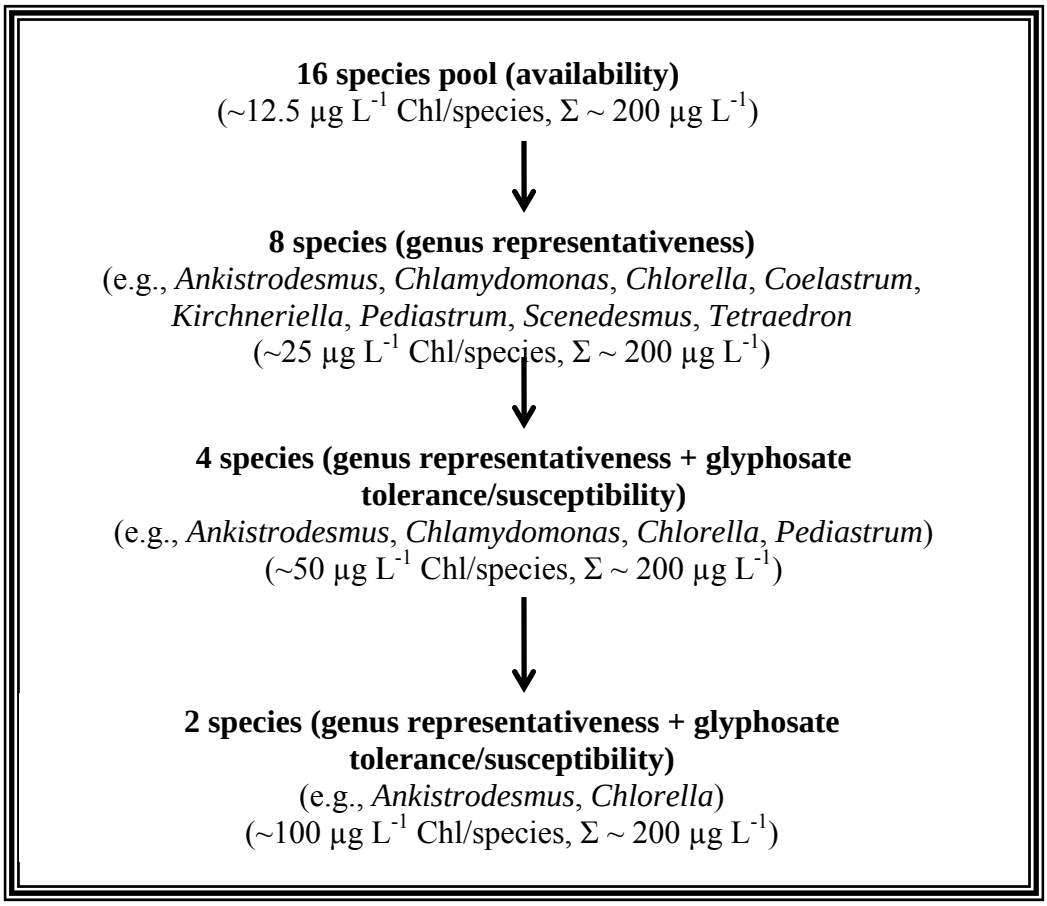

Figure 1. Schematic representation of the assemblage of communities based on genus representativeness and expected glyphosate tolerance/susceptibility. The communities were assembled with different numbers of algal species selected along a $\log_2$ richness gradient, but with the same initial total chlorophyll content (ca. 200 μg L^{-1} Chl). The contribution of Chl per species decreased gradually with the increase of the number of species in the communities (e.g., from 100 to 12.5 μg L^{-1}).

Phytoplankton counting

Samples for phytoplankton counting were fixed with Lugol's acetic-acid solution, stored in the dark at room temperature, diluted if needed, and settled (3 h per cm of chamber height) in Utermöhl sedimentation chambers of different volumes, depending on algal density. One or two vertical or horizontal stripes (ca. 120 fields of view/stripe) were observed at a magnification of 100× using an Olympus CK-40 (Japan) inverted microscope, and the number of cells of each species enumerated (Utermöhl, 1958). The population density (cells L^{-1}) of each species was calculated from counted cells and a factor, which included counted area, sedimented volume and % dilution. Cell volumes (biovolumes, μm^3) were calculated using the nearest geometrical shape (e.g., adapted sphere, inverted trapezoid, rotational ellipsoid,

cylinder or parallelepiped) that approximated the organism's shape (APHA-AWWA-WEF, 1999). At least five cells of the more common sizes were measured to obtain mean biovolume per species.

2.5 Data expression and calculation

Productivity of an assemblage was measured as NPP (net primary productivity), calculated as the rate of net chlorophyll accumulation after 7 and 14 days of incubation, and expressed in terms of μg chlorophyll day^{-1}.

Results

Productivity increased in all assembled communities in the first seven days of incubation and then declined (15% – 5×). Therefore, the analysis here was limited to data for the first seven days representing conditions for growth. The communities exposed to glyphosate were generally more productive (10–100 %) than their controls in a positive dose–dependent fashion, while the fenthion assay had no general pattern for the ranking of productivities among control and treatments (Figs. 2 and 3). Productivity levels were in average higher in the glyphosate batch I (50–140 μg Chl day^{-1}, Fig. 2) compared to the ones calculated for the subsequent batches (15–90 μg Chl day^{-1}, Figs. 2, 3), probably due to the influence of the replacement of *C. reinhardtii* by *C. botrytis*. The levels of productivity reached by the less productive assemblages in the glyphosate batch III and the fenthion II (2 spp.) (Fig. 2) were ~ 4× lower than their analogs in the glyphosate batch II and fenthion I.

The four species assemblages were the most productive in three of the five batches in spite of their different species composition (Fig. 2). This implies that the relationship between diversity and productivity for these batches was hump-shaped (concave), with an ascending part that peaked at a diversity of 4 species followed by a descending part that usually ended at the highest diversity (16 species). Only the Gly III assemblages in the batch III followed the expected positive asymptotic relationship between diversity and productivity with the saturation point situated at 4 spp., while the controls, and Gly I and II treatments in the batch I had the idiosyncrasy of presenting the expected asymptotic relationship after the hump, with the saturation point situated at 8 spp.. The controls in the batch II of glyphosate and the Fen II treatments in the fenthion I presented a saturated part between 4 and 8 spp.. Curiously, the control of the first of the two pooled batches that constituted the final glyphosate batch I presented the expected asymptotic relationship with the saturation point situated at 8 spp. (data not shown). No reductions in species numbers were recorded for these batches.

The two species assemblages were the most productive (and variable) in the glyphosate batch II (Fig. 3). They were hyper-dominated by *C. fusca* probably because the inoculates were made on chlorophyll basis and the *C. fusca* stock culture was producing less Chl than otherwise, so considerably more cells were added (personal observations). The treatments in this batch followed an inverse asymptotic pattern for the diversity–productivity relationship with minimums situated at a diversity of 4 spp., whereas the controls assemblages presented a decrease in productivity at the highest diversity (16 spp.). *Pediastrum boryanum* and *Tetraedron minimum*, which were co-dominating the assemblages in abundance at the beginning

of the bioassay (40 and 35 %) disappeared from the 8 spp. assemblages of the Gly III treatments without any impact on productivity.

The 16 spp. assemblages were the most productive in the fenthion batch I (Fig. 3). The diversity–productivity pattern was a hybrid between an inverse hump-shaped with the lowest productivity in the 4 spp. assemblages and a gradual rising (unimodal convex). The Fen III treatments, however, presented a rather saturated part where no major changes in productivity were observed with increasing diversity (2 to 8 spp.) until the increase at 16 spp. The idiosyncrasy of this batch was that no important changes in productivity were observed along the diversity gradient (< 20 %), except in the obvious case of the assemblages of 2 spp. of the Fen I treatments that only contained one species (*C. fusca*).

There was a prior effort made with glyphosate where we randomly assembled communities of 2, 5, 10 and 19 species, and hence with species composition different to the above mentioned batches that presented a similar pattern than the glyphosate batch I, but with productivity maximized at 5 spp. (data not presented). Curiously, the control presented the expected asymptotic relationship with the saturation point situated at 10 spp.. In general, this batch resembles the first batch but with higher productivities, especially in the most productive assemblages (Gly III treatments), probably due to the different species composition.

In order to identify potential species effects that could help us to explain the observed trends in the relationship diversity–productivity, we analyzed species interactions in terms of changes of relative abundances. For example, in the glyphosate batch I (Fig. 4) the control was the least productive possibly because only one species (*C. reinhardtii*) increased its contribution, while in the Gly III treatments the other three species but *C. fusca* increased their contribution. While in the batch II (Fig. 5), the Gly I treatment was the least productive probably because *A. braunii* increased its contribution until co-dominate the assemblage compared to the Gly III treatment where curiously *C. fusca* was dominating. In the batch III (Fig. 4), *K. cornuta* dominated the assemblage in the most productive Gly III treatment outcompeting *T. minimum*, while in the controls the assemblage tended to be more even.

In the 16 spp. assemblages of the fenthion batch I (Fig. 4), S. *acutus* f. *alternans* (a.f.a.) and *S. acuminatus* increased their contribution in the most productive control, while in the least productive assemblage, the Fen III treatment the other *Scenedesmus* (*obliquus)* was the main contributor. While in the 4 spp. assemblages of the batch II (Fig. 4), *K. cornuta* dominated the most productive control, while in the least productive Fen III treatment the same species was co-dominating with *C. microporum*. *C. fusca* was dominating the assemblages of 4 spp. assemblages in batch I (Fig. 5), but most importantly in the most productive Fen III treatment, while in the least productive control *P. boryanum* increased its contribution.

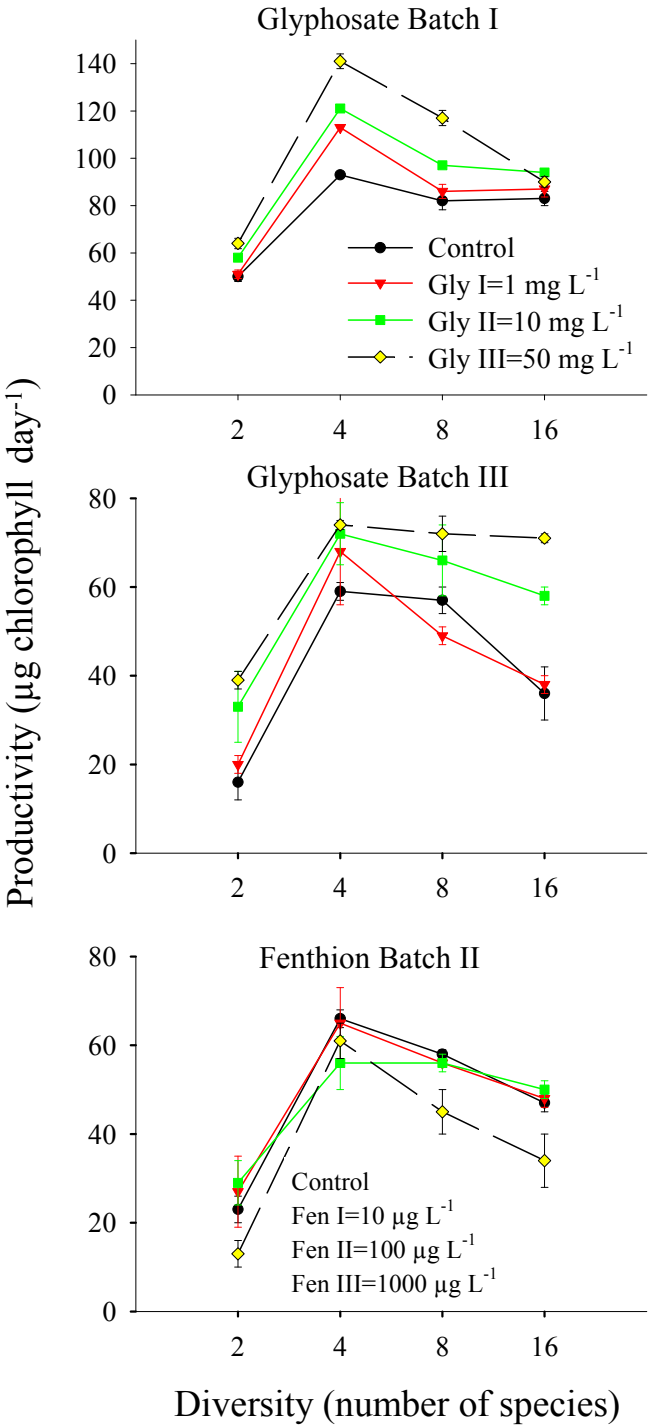

Figure 2. Relationship between diversity (as number of species) and net productivity (as chlorophyll in µg day^{-1}) in controls, glyphosate and fenthion treatments after seven days of incubation. The initial chlorophyll content was similar in all assemblages (~ 200 µg L^{-1}). The results for batch I of glyphosate is an average of two batches that were pooled, because species composition was identical. Data are connected by straight lines to highlight the form of the relationship. Mean of four replicates (glyphosate I and fenthion I) and two replicates (glyphosate III) is shown. Error bars represent the fractional standard deviation of the means.

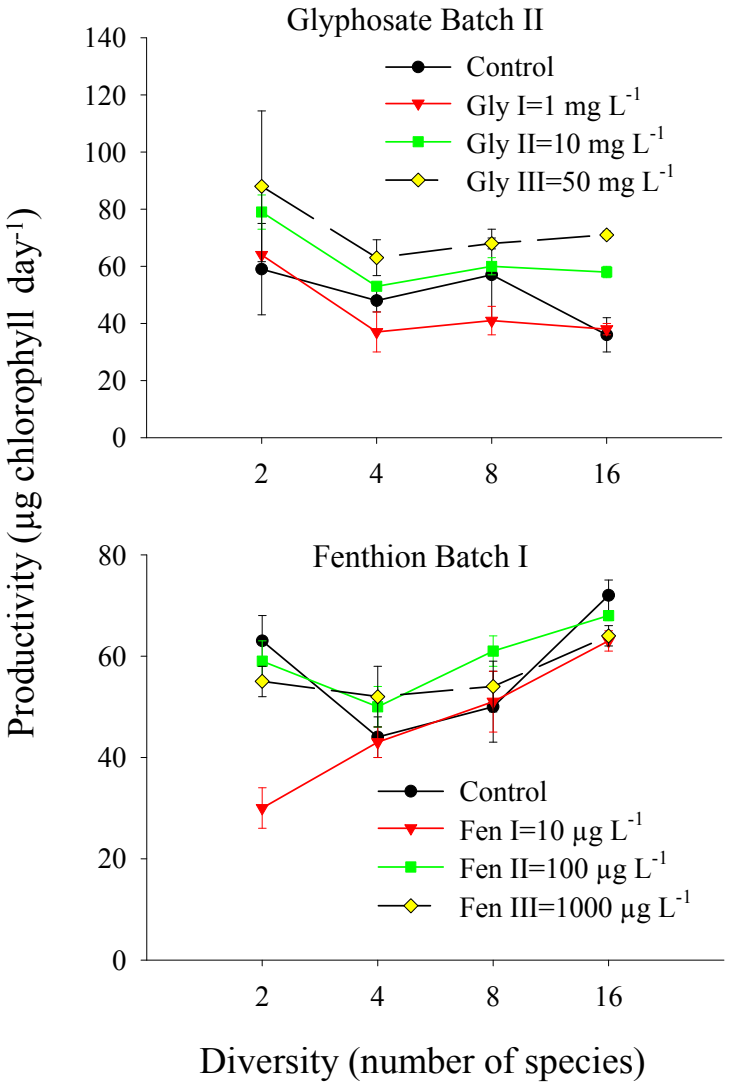

Figure 3. Relationship between (as number of species) and net productivity (as chlorophyll in µg day^{-1}) in controls, glyphosate and fenthion treatments after seven days of incubation. The initial chlorophyll content was similar in all assemblages (~ 200 µg L^{-1}), except in one of 2 spp. (Fen I) in the fenthion batch that only contained one species (*Chlorella fusca*). Communities of 2 spp. in the glyphosate batch were hyperdominated by *C. fusca*. Two species went extinct from the Gly III assemblages of 8 spp. in the glyphosate batch. Data are connected by straight lines to highlight the form of the relationship. Mean of two replicates for the glyphosate batch and four replicates for the fenthion batch is shown. Error bars represent the fractional standard deviation of the means.

From the changes in abundance we also conclude that at least three species seem to be favoured by glyphosate: *A. braunii* directly, and *K. cornuta* and *C. microporum* indirectly *via* competition. *T. minimum*, *P. boryanum* and *S. quadricauda* seem to be directly affected and *C. reinhardtii* indirectly *via* competition. *C. fusca* sometimes was disfavoured and others favoured. Fenthion, on the other hand affected directly to *S. quadricauda*, *C. fusca*, *S. capricornutum*, and to some extent *S. acutus* f. *alternans* and *S. acuminatus*, and indirectly through competition to *K. cornuta* and *P. boryanum*. Four species seem to tolerate it: *S. obliquus, K. lunaris*, *T. minimum*, *C.*

microporum and to a minor extent *A. braunii*. *C. fusca* presented intermediate tolerance because in one batch was tolerant and in the other sensitive.

C. reinhardtii was expected to be tolerant to glyphosate and resulted to be a bad competitor in the ability to use it as a nutrient source, which also applies to *C. fusca*. On the other hand, *S. quadricauda* that was expected to be susceptible to glyphosate resulted to be also a poor competitor in competing for it, and *A. braunii* that was expected to have intermediate tolerance resulted to be a good competitor for glyphosate. *C. fusca* presented an intermediate tolerance to fenthion as expected.

Discussion

Our general result does not support any specific hypothesis of the relationship between diversity and productivity, but a hybrid of hypotheses including the diversity-productivity hypothesis, the redundant hypothesis, and the idiosyncratic hypothesis, regardless of species composition, hence showing unimodal and saturating parts instead of monotonic relationships. This hump-shaping of the relationship probably happened due to competition for limiting resources in our initially dense communities that probably limit the environment carrying capacity to a number of available roles at 4 species to maximize productivity. However, the maximized productivity obtained in the first attempt with glyphosate at 5 spp. and the replicated result of diminished productivity at 8 spp. might imply the possibility that our systems could carry maximized productivities with species numbers between 4 and 7 spp. This is in agreement with observations of low primary producer diversity in many high productivity ecosystems around the world, including algal blooms, salt and freshwater marshes, riparian forests in the tropics and temperate zones (Huston and McBride, 2004). Experimental studies made with herbaceous plants suggest that productivity is a decelerating function of species richness, often saturating at low species numbers (1-5 species) (Tilman et al., 1997; Mittelbach et al., 2001; Naeem et al., 2004). However, in other experiments different relationships between species richness and above-ground biomass production were found at eight European grassland sites: linear for Germany and Sweden, log linear for Portugal and Switzerland, quadratic for Sheffield, and non-significant for Greece (Hector et al., 2004). The productivity differed from site to site even when the communities had the same species or mixtures of species. Hector and Bagchi (2007) found that between 3 and 7 species were sufficient to perform each process of the seven studied in a recent ecosystem multifunctionality analysis. However, because different species often influence different processes, greater biodiversity than suggested by studies focusing on single ecosystem processes in isolation will be required to maintain multifunctional ecosystems.

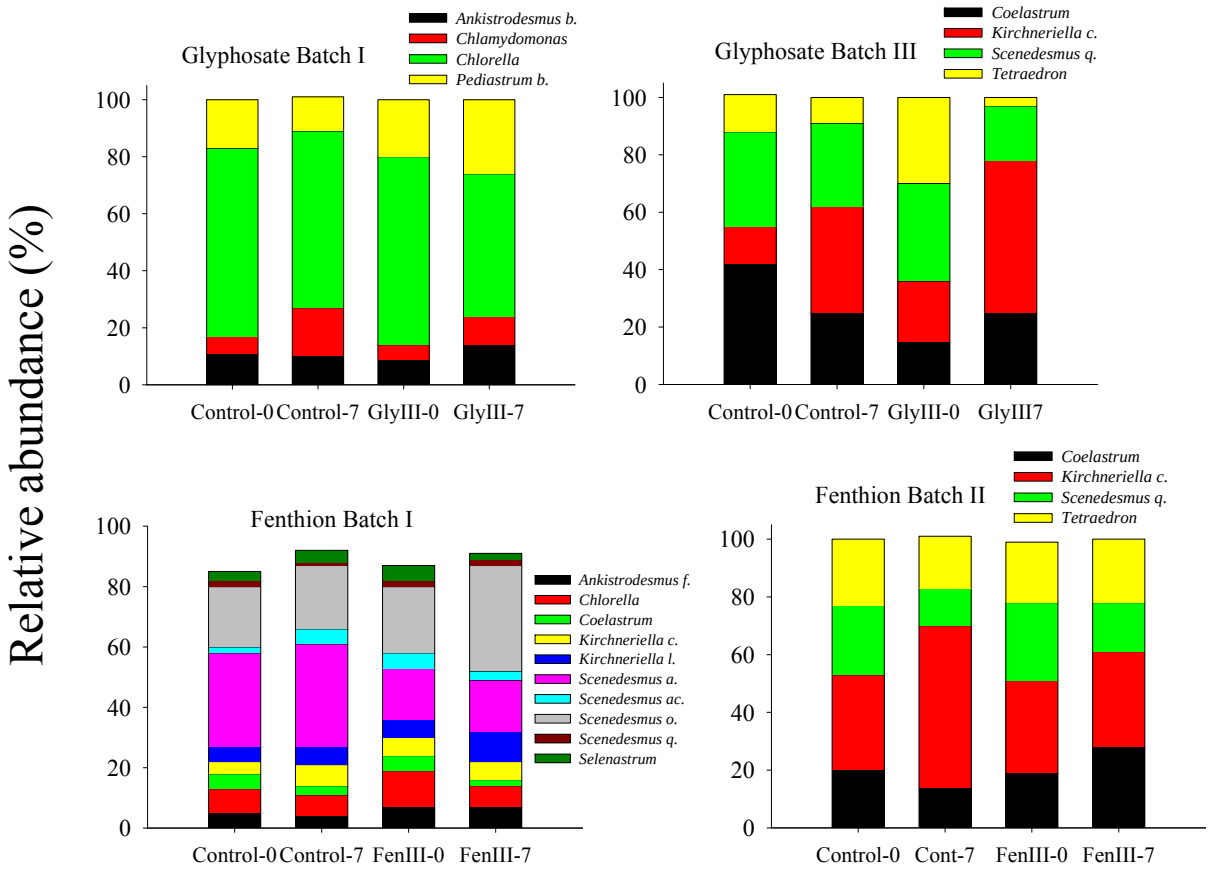

Figure 4. Time trends in average relative abundance in controls and highest concentration treatments (Gly III= 50 mg L^{-1}, and Fen III= 1000 µg L^{-1}) in batches I and III of glyphosate (4 spp.), and I and II of fenthion (16 spp. and 4 spp., respectively), at the beginning (0) and after 7 days of incubation. For the first batch of fenthion, only the ten species with the most important contributions are presented.

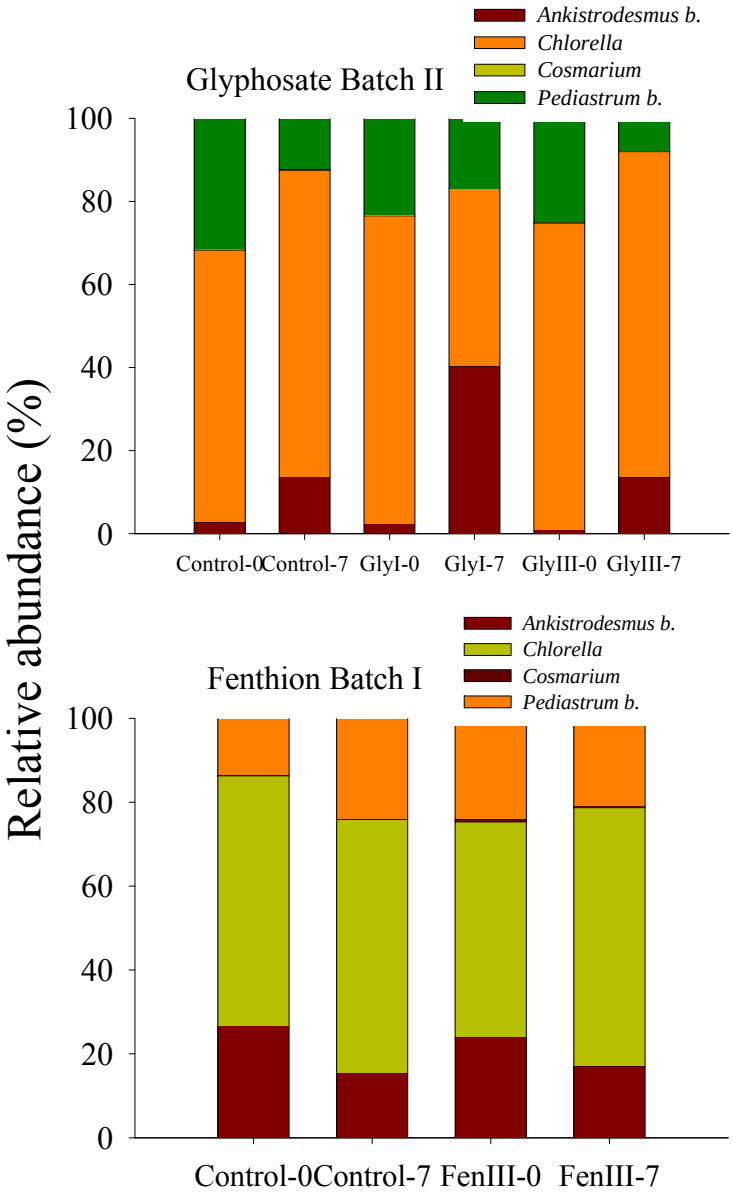

Figure 5. Time trends in average relative abundance in controls and highest concentration treatments (Gly III= 50 mg L^{-1}, and Fen III= 1000 µg L^{-1}) in the 4 spp. assemblages of batches glyphosate II and fenthion I, at the beginning (0) and after 7 days of incubation. The results from the Gly I treatment (1 mg L^{-1}) are also presented given that was the least productive of all the treatments and had different patterns. *Cosmarium botrytis* made a very low contribution in abundance throughout both bioassays and hence its presence is not obvious.

The species-specific impacts on the diversity–productivity relationship, on the other hand, were the hidden treatments in the bioassays, and highlighted the important roles of algae-bacteria associations (fenthion batch I and glyphosate batch II, Fig. 3, *C. botrytis* case) and dominance (glyphosate batch II, Fig. 3, *C. fusca* case). A hidden treatment is any factor that is correlated with the treatments in an experiment, but not explicitly controlled, manipulated, measured, or considered in the experimental design and unless identified may lead to misinterpretation of results (Huston 1997). For example, the numerical hyperdomination by *C. fusca* in the second batch of glyphosate, allowed that the 2 spp. were the most productive assemblages flattening the relationship afterwards. On the other hand, the bacteria associated with the non-axenic culture of *C. botrytis* were capable of switching the concave relationship to a convex relationship, most likely by enhancing resource exploitation and competing with algae (including its host), due to the large portion of total uptake of inorganic nutrients (phosphate and ammonium) that they account for (Kirchman, 1994), and hence reducing algal productivity (Loreau et al., 2001). As a result of the interference with bacteria, a minimum rather than a maximum occurred at 4 spp. and in general, buffered the impact of diversity on productivity. However, the competitive effect of bacteria on algal productivity was slightly outweighed at the highest diversity (16 spp.) giving credit to the idea that biodiversity acts as a "biological insurance" against the disruptive effects of environmental fluctuations on ecosystem functioning (Loreau, 2000; Hector and Bagchi, 2007). The important role of primary producer-microbe interaction on the relationship between diversity and productivity has been recognized in algae-bacteria and arbuscular mycorrhizal (AMF) associations and usually explained by nutrient availability dynamics (Klironomos et al., 2000; Naeem et al., 2000; Van Der Heijden and Cornelissen, 2004). In microcosms experiments when bacteria were not added, algal production was positively associated with species richness, but when bacteria were added, algal production was significantly affected by the interaction between algal species richness and decomposer species richness (Naeem et al., 2000). The pattern of the relationship diversity–productivity was very sensitive to the addition of decomposers presenting maximums and asymptotes depending on the number of bacterial species added (Petchey et al., 2004). Klironomos et al. (2000), on the other hand, concluded that in the absence of AMF, the relationship diversity-productivity in plants was positive and linear within the 15 species range tested supporting the diversity-productivity hypothesis, whilst in the presence of AMF the relationship was asymptotic and saturated at a diversity of 2 spp. hence supporting the redundancy hypothesis. The saturation was explained by competition due to a reduction in the amount of resources available to plant communities in the presence of AMF, which exert a very high carbon demand compared with non-AMF systems.

Niche complementarities, competition, and co-existence may provide mechanistical explanations to the hybrid patterns of the diversity–productivity relationship. A positive relationship between diversity and productivity is often interpreted as evidence for the operation of diversity-promoting interactions among many species, for example, when niche complementarity is operative and dominates competition, assuming that each species utilizes a resource at a higher intensity as diversity increases (Tilman et al., 1997; Loreau, 1998; Hector et al., 2004; Hille Ris Lambers et al., 2004). However, as species diversity continues to increase, the probability that species will overlap in their resource use increases, thus creating a decelerating relationship, the steepness of which may depend on the strength of

competition (Bond and Chase, 2002). Asymptotes of the response of primary productivity to species diversity may depend on species redundancy and invariance of the average resource-use intensity with diversity, hence promoting coexistence (Loreau, 2000; Mouquet et al., 2002).

These general hypotheses and insights can be used to interpret the patterns in Figs. 2 and 3 by a verbal model: *i*) an ascending part may imply the absence of competition for resources (i.e., niche complementarity), e.g., the change from to 2 to 4 spp. in the three batches (Fig. 2), *ii*) asymptotes may imply redundancy and mechanisms that promote coexistence, e.g., from 8 to 16 spp. in the first batch of glyphosate (Fig. 2), and *iii*) a descending part might imply complete competition for resources with steepness depending on the degree of competition, e.g., changes from 4 to 8 spp. in the third batch of glyphosate and the second of fenthion (Fig. 2). Glyphosate when dosed at 50 mg L^{-1}, however sometimes acted as an instigator or provoker for complete competition (e.g., from 4 spp. to 16 spp in the first batch), while as a buffer for competition in the third batch (Fig. 2). In the *C. fusca* hyperdominated second batch of glyphosate (Fig. 3), the herbicide buffered competition between 8 and 16 spp. at all assayed concentrations. In the first batch of fenthion, the highest treatment (1000 µg L^{-1}) buffered both competition and niche complementarity producing less steep parts (asymptotes) in the relationship diversity–productivity compared to the controls and other treatments (Fig. 3).

Glyphosate did not change the trajectory of the relationship at the lowest and intermediate diversities, but at the highest diversities acting usually as a buffer for competition (batches II and III) and others as a provoker (Gly III, batch I). The herbicide had an important impact on the magnitude of the effect of diversity on productivity sharpening the relationship probably by subsidizing it at almost all assayed concentrations including its EEC for aquatic exposure and especially at the highest concentration (50 mg L^{-1}). This was partially expected because the herbicide may be used as a nutrient source and produce stimulatory effects in primary producers when dosed at low concentrations (Wong, 2000; Paper III, Cedergreen et al., 2007; Velini et al., 2008), but this does not apply to the highest assayed concentration that is around the EC50 of the standard algal test species. This was probably influenced by the high initial density of the cultures, which might have caused important biodilution of the herbicide (Paper III). The results obtained in two attempts (data not shown), however, brings out the possibility that glyphosate may have the ability to saturate productivity at lower levels of diversity (shifting the hump from 8–10 spp. to 4–5 spp.) and change the form of the relationship from asymptotic to unimodal, probably due to its potential to be utilized as a nutrients source. *Pediastrum boryanum* and *Tetraedron minimum* were not found in one assemblage treated with the highest concentration with no obvious impact on productivity.

Fenthion as expected had a lower impact on the effect of diversity on productivity than the one exerted by glyphosate because it is an insecticide, did not cause species extinctions, and also did not greatly change the pattern of the relationship. However, the insecticide tended to diminish productivity when dosed at concentrations around but lower than NOEC and especially around EC50 values of single species (100 and 1000 µg L^{-1} respectively) in the most productive assemblages (batch I and II), and to increase it at intermediate levels of diversity at the same concentrations (batch I). Fenthion is a hydrophobic compound and expected to affect

algae only at high concentrations (~1000 μg L^{-1}) (USEPA, 1998; Yeh and Chen, 2006), most likely due to its disorganization of chloroplasts (Yukimoto, 1983). This suggests that the relationship is more responsive (sensitive) to fenthion than single species toxicity tests. This suggests that the relationship is more responsive (sensitive) to glyphosate and fenthion than single species toxicity tests (No Observed Effect Concentration, NOEC =11 mg/L and 200 μg/L, respectively).

Species composition, on the other hand, did make an impact in the level of productivity reached in the assemblages hence affecting the amplitude of the relationship diversity–productivity, probably through inclusion or exclusion of productive or susceptible/tolerant species. For example, the most productive assemblages in first batch of gyphosate (4 spp.) had the highest productivity of all batches, most probably due to the presence of *C. reinhardtii*.

In conclusion, this study shows that the relationship between diversity and productivity is unimodal (concave) at low levels of diversity, asymptotic at higher levels, and independent of species composition for algal communities under competition for acquisition of limiting resources, having maximums (or minimums) and saturated parts which might be context-dependent and explained by mechanisms that govern the nature and strength of species interactions. Productivity was maximized at a diversity of 4 species, but with potential to be between 4 and 7 spp. Pesticides did not greatly change the trajectory of the relationship but its amplitude, which implies an impact on the magnitude of the effect of diversity on productivity. For example, the herbicide glyphosate produced a sharpening in the relationship, acting as a nutrient source and hence subsidizing productivity at almost all assayed concentrations, including its EEC for aquatic exposure and especially at the highest tested concentration. Fenthion did not affect the amplitude of the relationship at the same extent that glyphosate, but impacted the effect of diversity on productivity at concentrations above its EEC for aquatic exposure. Algae–bacteria associations and hyperdominance of a single species turned the diversity–productivity pattern up side down, or widened and flattened it. Species composition did make an impact in the level of productivity reached in the assemblages hence affecting the amplitude of the relationship diversity–productivity, probably through inclusion or exclusion of productive or susceptible/tolerant species. Our results suggest that declining and augmenting diversity may result in local changes in productivity that might be enhanced or buffered by pesticides.

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Appendix: Tables

Table 1. List of algal species of the class Chlorophyceae used in the experiments, with designation numbers given by UTEX (University of Texas) and the corresponding code given by us (CIRA Nr.)

Species & auctor	UTEX Nr.	CIRA Nr.
Ankistrodesmus braunii Brunnthaler	187	1
Ankistrodesmus densus Korsh.	190	2
Ankistrodesmus falcatus (Corda) Ralfs.	749	3
Chlamydomonas reinhardtii Dang. [E&S]	2246	6
Chlorella fusca var. *fusca* Shihira et Krauss [E&S]	343	10
Coelastrum microporum Näg.	281	11
Cosmarium botrytis Menegh.	LB953	12
Kirchneriella cornuta Korsh.	LB1355	15
Kirchneriella lunaris (Kirch.) Moebius	285	16
Pediastrum boryanum var. *cornutum* (Racib.) Salek	LB470	18
Pediastrum simplex (Meyen) Lemm.	LB1601	19
Scenedesmus acutus f. *alternans* Hortobagyi	72	21
Scenedesmus acuminatus (Lagerh.) Chod.	415	22
Scenedesmus obliquus (Turp.) Kütz.	78	24
Scenedesmus quadricauda (Turp.) Bréb.	76	25
Selenastrum capricornutum Printz	1648	26
Tetraedron minimum var. *scrobiculatum* Lagerh.	LB1371	28

Table 2. Species pool used to assemble algal communities with a diversity gradient (as levels in species numbers) in experimental batches, and expected degree of susceptibility/tolerance to glyphosate and fenthion

Pesticide / Batch No./ (No. of replicates)	2 species assemblages (CIRA Nr.)	4 species assemblages (CIRA Nr.)	8 species assemblages (CIRA Nr.)	16 species assemblages (CIRA Nr.)
Glyphosate I (4)	1, *10*	1, *6*, *10*, 18	1, *6*, *10*, 11, 15, 18, **25**, 28	1, 2, 3, *6*, *10*, 11, 15, 16, 18, 19, **21**, 22, 24, **25**, 26, 28
Glyphosate II (2)	*10*, 12	1, *10*, 12, 18	1, *10*, 11, 12, 15, 18, **25**, 28	1, 2, 3, *10*, 11, 12, 15, 16, 18, 19, **21**, 22, 24, **25**, 26, 28
Fenthion I (4)	1*,10	1*, 10, 12, 18	1*, 10, 11, 12, 15, 18, 25, 28	1*, 2, 3, 10, 11, 12, 15, 16*, 18, 19, 21, 22, 24, 25, 26†, 28
Glyphosate III (2)	1, 18	11, 15, **25**, 28	2, 3, 16, 19, **21**, 22, 24, 26	Idem to Glyphosate II
Fenthion II (4)	1*, 18	11, 15, 25, 28	2, 3, 16*, 19, 21, 22, 24, 26†	Idem to Fenthion I

Bold: susceptible to glyphosate (Sáenz et al 1997, Wong 2000)
Italic: tolerant to glyphosate (Maule and Wright 1984, Anton et al 1993, Faust et al 1993, Reboud 2002)
Underlined: intermediate susceptibility to glyphosate (Bozeman et al 1989, Gardner et al 1997, Ma 2002)
*: tolerant to 50 µg L^{-1} fenthion (~Fen I) and susceptible to 500 µg L^{-1} (~Fen III) (Hanazato and Kasai 1995)
†: tolerant to 300–700 µg L^{-1} fenthion (Fen I, II), EC50 ≥ Fen III (USEPA 1998, Yeh and Chen 2006)
Others: unreported susceptibility/tolerance to glyphosate or fenthion

References

APHA-AWWA-WEF, 1999. Standard methods for the examination of water and wastewater. 20th Edition. American Public Health Association, American Water Works Association, Water Environment Federation.

Battaglin, W. A., Kolpin, D. W., Scribner, E. A., Kuivila, K. M., and Sandstrom, M. W., 2005. Glyphosate, other herbicides, and transformation products in midwestern streams, 2002. J. Am. Water Resour. Assoc. 41, 323-332.

Baylis, A. D., 2000. Why glyphosate is a global herbicide: strenghts, weaknesses and prospects. Pest Manag. Sci. 56, 299-308.

Bengtsson, J., Engelhardt, K., Giller, P., Hobbie, S., Lawrence, D., Levine, J., Vilá, M., and Wolters, V., 2004. Slippin' and slidin' between the scales: the scaling components of biodiversity- ecosystem functioning relations. In Loreau, M., Naeem, S., Inchausti, P. (Eds.), Biodiversity and ecosystem functioning: synthesis and perspectives, Oxford University Press, pp. 209-220.

Bond, E. M., and Chase, J. M., 2002. Biodiversity and ecosystem functioning at local and regional spatial scales. Ecol. Lett. 5, 467-470.

Cairns, J. J., Niederlehner, B. R., and Smith, E. P., 2003. Chapter 19: Ecosystem effects: functional end points. In Rand, G. M. (Eds.), Fundamentals of aquatic toxicology: effects, environmental fate and risk assessment, Taylor & Francis, London, pp. 589-607.

Cedergreen, N., Streibig, J. C., Kudsk, P., and Mathiassen, S. K., 2007. The ocurrence of hormesis in plants and algae. Dose Response 5, 150-162.

Clements, W. H., and Newman, M. C., 2002. Community ecotoxicology. John Wiley & Sons Inc., Chichester.

Ford, J., 1989. The effects of chemical stress on aquatic species composition and community structure. In Levin, S. A., Harwell, M. A., Kelly, J. R. and Kimball, K. D. (Eds.), Ecotoxicology: problems and approaches, Springer-Verlag, New York, pp. 99-144.

Giller, P. S., and O'Donovan, G., 2002. Biodiversity and ecosystem function: do species matter? Proc. R. Ir. Acad [B] 102B, 129-139.

Hector, A., and Bagchi, R., 2007. Biodiversity and ecosystem multifunctionality. Nature 448, 188-191.

Hector, A., Loreau, M., Schmid, B., and Biodepth, 2004. Biodiversity manipulation experiments: studies replicated at multiple sites. In Loreau, M., Naeem, S. and Inchausti, P. (Eds.), Biodiversity and ecosystem functioning: synthesis and perspectives, Oxford University Press, pp. 36-46.

Hille Ris Lambers, J., Harpole, W. S., Tilman, D., Knops, J., and Reich, P., 2004. Mechanisms responsible for the positive diversity-productivity relationship in Minnesota grasslands. Ecol. Lett. 7, 661-668.

Huston, M. A., and McBride, A. C., 2004. Evaluating the relative strengths of biotic versus abiotic controls on ecosystem processes. In Loreau, M., Naeem, S., Inchausti, P. (Eds.), Biodiversity and ecosystem functioning: synthesis and perspectives, Oxford University Press, pp. 47-60.

Jespersen, A. M., and Christoffersen, K., 1987. Measurements of chlorophyll a from phytoplankton using ethanol as extraction solvent. Arch. Hydrobiol. 109, 445-454.

Kirchman, D. L., 1994. The uptake of inorganic nutrients by heterotrophic bacteria. Mic. Ecol. 28, 255-271.

Klironomos, J. N., McCune, J., Hart, M., and Neville, J., 2000. The influence of arbuscular mycorrhizae on the relationship between plant diversity and productivity. Ecol. Lett. 3, 137-141.

Levine, S. N., 1989. Theoretical and methodological reasons for variability in the responses of aquatic ecosystem processes to chemical stresses. In Levin, S. A., Harwell, M. A., Kelly, J. R. and Kimball, K. D. (Eds.), Ecotoxicology: problems and approaches, Springer-Varlag, New York, pp. 145-179.

Loreau, M., 1998. Biodiversity and ecosystem functioning: a mechanistical model. Proc. Natl. Acad. Sc. U.S.A. 95, 5632-5636.

Loreau, M., 2000. Biodiversity and ecosystem function: recent theoretical advances. Oikos 91, 3-17.

Loreau, M., 2004. Does functional redundancy exist? Oikos 104, 606-611.

Loreau, M., Naeem, S., Inchausti, P., Bengtsson, J., Grime, J. P., Hector, A., Hooper, D. U., Huston, M. A., Rafaelli, D., Schmid, B., Tilman, D., and Wardle, D. A., 2001. Biodiversity and ecosystem functioning: current knowledge and future challenges. Science 294, 804-808.

Mittelbach, G. G., Steiner, C. F., Scheiner, S. M., Gross, K. L., Reynolds, H. L., Waide, R. B., Willig, M. R., Dodson, S. I., and Gough, L., 2001. What is the observed relationship between species richness and productivity? Ecology 82, 2381-2396.

Mouquet, N., Moore, J. L., and Loreau, M., 2002. Plant species richness and community productivity: why the mechanisms that promotes coexistence matters. Ecol. Lett. 5, 56-65.

Naeem, S., Hahn, D. R., and Schuurman, G., 2000. Producer-decomposer co-dependency influences biodiversity effect. Nature 403, 762-764.

Naeem, S., Loreau, M., and Inchausti, P., 2004. Biodiversity and ecosystem functioning: the emergence of a synthetic ecological framework. In Loreau, M., Naeem, S., and Inchausti, P. (Eds.), Biodiversity and ecosystem functioning: synthesis and perspectives, Oxford University Press, pp. 3-11.

Odum, E. P., 1985. Trends expected in stressed ecosystems. BioScience 35, 419-422.

Petchey, O. L., Morin, P. J., Hulot, F. D., Loreau, M., McGrady-Steed, J., and Naeem, S., 2004. Contributions of aquatic model systems to our understanding of biodiversity and ecosystem functioning. In Loreau, M., Naeem, S., Inchausti, P. (Eds.), Biodiversity and ecosystem functioning: synthesis and perspectives, Oxford University Press, pp. 127-138.

Pratt, J. R., and Cairns, J. J., 1996. Chapter 12: Ecotoxicology and the redundancy problem; understanding effects on community structure and function. In Newman, M. C. and Jagoe, C. H. (Eds.), Ecotoxicology: a hierarchical treatment, CRC Press, Inc., Boca Ratón, pp. 347-370.

Schindler, D. W., 1987. Detecting ecosystem responses to anthropogenic stress. Can. J. Fish Aq. Sci. 44, 6-25.

Sheehan, P. J., 1984. Functional changes in the ecosystem. In Sheehan, P. J., Miller, D.R., Butler, G.C., and Bourdeau P. (Eds.), Effects of pollutants at the ecosystem level, Scientific committee on problems of the environment (SCOPE), Norwich, pp. 101-145.

Stevenson, R. J., and Smol, J. P., 2003. Use of algae in environmental assessments. In Wehr, J. D. and Sheath, R. G. (Eds.), Freshwater algae of North America, ecology and classification, Academic Press, San Diego, pp. 775-804.

Tilman, D., Knops, J., Wedin, D., Reich, P., Ritchie, M., and Siemann, E., 1997. The influence of functional diversity and composition on ecosystem processes. Science 277, 1300-1305.

Tomlin, C., 1997. The pesticide manual. British Crop Protection Council, Surrey.

Trenbath, B. R., 1974. Biomass productivity of mixtures. Adv. Agron. 26, 177-210.

USEPA, 1993. Methods for measuring the acute toxicity of effluents to freshwater and marine organisms. U.S. Environmental Protection Agency EPA/600/4-90/027F, Cincinnati, OH.

USEPA, 2000. Pesticide ecotoxicity database. U. S.Environmental Protection Agency, Washington D.C.

USEPA, 1998. Reregistration eligibility decision Fenthion. EFED chapter. Case 0290. U. S. Environmental Protection Agency

Utermöhl, H., 1958. Zur wervollkommung der quantitative phytoplankton metodic. Arch. Hydrobiol. 9, 1-38.

Van Der Heijden, M. G. A., and Cornelissen, J. H. C., 2004. The critical role of plant-microbe interactions on biodiversity and ecosystem functioning: arbuscular mycorrhizal associations as an example. In Loreau, M., Naeem, S., Inchausti, P. (Eds.), Biodiversity and ecosystem functioning: synthesis and perspectives, Oxford University Press, pp. 181-192.

Vandermeer, J., 1989. The ecology of intercropping. Cambridge University Press, Cambridge.

Velini, E. D., Alves, E., Godoy, M. C., Meschede, D. K., Souza, R. T., and Duke, S. O., 2008. Glyphosate applied at low doses can stimulate plant growth. Pest Manag. Sci. 64, 489-496.

Wong, P. K., 2000. Effects of 2,4-D, glyphosate and paraquat on growth, photosynthesis and chlorophyll-a synthesis of *Scedesmus quadricauda* Berb 614. Chemosphere 41, 177-182.

Yeh, H. J., and Chen, C. Y., 2006. Toxicity assessment of pesticides to *Pseudokirchneriella subcapitata* under air-tight test environment. J. Haz. Mat. A131, 6-12.

Yukimoto, M., 1983. Effect of organophosphorus insecticides on Hill reaction. J. Pest. Sci. 8, 63-68.

Contribution of groundwater residence time and biodegradation to persistence and effects of pesticides in aquifers

Katia Montenegro[1, 2], Gerhard Barmen[3] and Göran Bengtsson[1]
[1]Department of Ecology, Lund University, SE-22362, Lund, Sweden.
[2]CIRA-UNAN/Managua, Apartado Postal 4598, Managua, Nicaragua.
[3]Engineering Geology, Lund University, P.O.Box 118, SE-22100, Lund, Sweden.

Abstract

Anthropogenic substances are found in aquifers around the world and cause concern on groundwater quality and toxic effects on microbiota because of long residence times of the groundwater. Microbial processes play important roles in nutrient cycling (e.g., nitrification and denitrification) and natural attenuation of contaminants (biodegradation) in groundwater where they may be the sole means of transformation. Our general objective was to estimate the contribution of residence time of groundwater and *in situ* biodegradation potential to the persistence and effects of contaminants in aquifers of the selected area of León-Chinandega. To fulfill the objective we collected ground waters to provide natural inoculates for the biodegradation, nitrification and denitrification assays, and to be analyzed for water stable isotopes (Oxygen-18 and Deuterium) and chlorofluorocarbons to determine recharge sources and apparent ages. The isotopic results show that the deep well predominantly recharged at higher elevations (500 masl), meanwhile the shallow wells recharge at lower elevations (100 to 350 masl) and that ground waters in the area are mixtures of water infiltrated at the plains and at the volcanic slopes. For example, the ground waters in the plains receive an equal contribution of recharge from high and low altitudes. The apparent age of ground waters is in average 23 years with a range from 16 to 29 years. The youngest ages derived from CFC-11 concentrations at all studied sites indicate potential degradation of the chlorofluorocarbon, especially in the discharge area and most likely due to the poor oxygen conditions. Both the pristine deep aquifer and especially the shallow contaminated aquifer have degradation potential for DDE and glyphosate, and the most contaminated site may have strains adapted for faster degradation. The moderately contaminated sites had the highest potentials for nitrification and denitrification, and the most heavily contaminated the lowest for nitrification, most probably due to the presence of toxaphene. Denitrification was also responsive to contamination but at less extent than nitrification. The persistence of the *in situ* contaminants is not explained by the apparent age of water or the provenience. A combination of age tracers should be used to resolve the age distribution of ground waters in the area, due to the complexity of flow paths and mixing of waters.

Introduction

Contamination of groundwater by solutes such as, aromatic hydrocarbons, pesticides, and their metabolites, and nitrate, is common all over the world and especially in agricultural areas, rising concern about ecological and human health effects (Aamand et al., 1989; Johnston et al., 1998; Böhlke, 2002; Lapworth and Goody, 2006; Osenbrück et al., 2006; Thayalakumaran et al., 2008).

The persistence and fate or organic contaminants in groundwater largely depends on their rate of degradation (Washington, 2001) and the residence time or age of groundwater, which is the mean travel time of water from the point of recharge to the point of collection. The residence time can be used to reconstruct past releases of contaminants to aquifers besides its traditional use to characterize flow (Solomon et al., 1998; Bockgård et al., 2004). Dissolved gases have been used as tracer of water residence times, among them, chlorofluorocarbons, especially for dating of young ground waters < 45 y or to indicate whether the water contains a fraction of post-1940's water (Busenberg and Plummer, 1992; Focazio et al., 1998; Solomon et al., 1998). The fate at the same time is a key parameter for estimating the risk of long-term adverse effects on biota (OECD, 2006). Biological processes may modify organic molecules at the site of their discharge or during their transport, and in some environments as groundwater, are the sole means of natural attenuation (Alexander, 1994). For example, aromatic hydrocarbons, pesticides and nitrate in ground waters may induce acclimatization or adaptation in indigenous bacterial communities, hence increasing the rate of natural attenuation of contaminants (Aamand et al., 1989; Cavalier et al., 1991; Bengtsson and Bergwall, 1995).

The turnover of nitrogen is an important determinant of water quality and sustainable fertility (Stevenson, 1982; Pell et al., 1998; Pauer and Auer, 2000; Kemp and Dodds, 2002), and the autotrophic aerobic nitrification and the anaerobic denitrification are the most commonly used endpoints in ecological risk assessment of contaminants (Van Beelen and Doelman, 1997; Pell et al., 1998). Nitrification transforms less mobile soil nitrogen (ammonia) into a mobile form (nitrate) (Pell et al., 1998), and explaining why nitrate is the most ubiquitous groundwater contaminant in the world today (Böhlke, 2002; Thayalakumaran et al., 2008). Denitrification is the major biological process that account for nitrogen loss to the atmosphere (natural attenuation) hence has stimulated research given the ubiquitous nitrate pollution in groundwater from agricultural areas (Stevenson 1982, Pell et al. 1998). In general, nitrification is a more sensitive endpoint in risk assessment than denitrification (Domsch et al., 1983; Van Beelen and Doelman, 1997).

Anthropogenic substances are found in shallow as well as deep aquifers (Lapworth and Goody, 2006; Osenbrück et al., 2006; Taylor et al., 2006) and causes concern about drinking water quality and toxic effects on remote and pristine ecosystems because of long residence times of the groundwater. Much effort has been directed towards understanding how the contaminants are transported from the land surface to the aquifers, how they move within and between aquifers, and how their fate may be predicted even when the flow fields are heterogeneous in hydrology, physics and chemistry. Transport of contaminants from continuous anthropogenic sources on the land surface may then explain their persistence in deep aquifers. However, it is also possible that contaminants persist in deep aquifers due to the long residence times of water and slow biodegradation in oligotrophic communities in which few species are adapted to degrade anthropogenic substances. We focus this work on the latter and test whether the pattern of distribution of pesticides in selected ground waters in

the province of Chinandega in Nicaragua is related to the pattern of groundwater age and provenience and to the pattern of biodegradation and effects of pesticides on the indigenous microbial community. Persistent organochlorine pesticides, their metabolites, and nitrates, at varying concentrations ranging from background to heavily polluted, have been observed in wells in the province (Dahlberg and Odebjer, 2002; Delgado, 2003; Moncrieff et al., 2008) and it has been suggested that mainly the shallowest flow system is affected by intensive agricultural activity and human settlement in the area (Dahlberg and Odebjer, 2002; Delgado, 2003; Moncrieff et al., 2008). Environmental tracers (^{18}O and tritium) have shown mixing of groundwater originating from different elevations (Payne and Yurtsever, 1974; Dahlberg and Odebjer, 2002; Delgado, 2003).

Our general objective was to estimate the contribution of residence time of groundwater and *in situ* biodegradation potential to the persistence and effects of contaminants in aquifers of the selected area of León-Chinandega. The specific objectives were to determine groundwater residence time, potential half-lives of selected contaminants, and toxicity of the *in situ* contamination on potential nitrification and denitrification in aquifers feeding selected sites from the study area. To fulfill these objectives we estimated the age (apparent), and provenience of groundwater along flow paths (i.e., recharge areas, plains and discharge), and along transects with a gradient of pesticide-contamination. This was combined with an effort to describe their relationship with the potential half-lives of selected contaminants (the insecticide metabolite pp'-DDE and the herbicide glyphosate), and with the actual impact of contamination on microbial processes measured by ecotoxicological effects on potentials for nitrification and denitrification. Ground waters were collected to provide natural inoculates for the biodegradation, nitrification and denitrification assays, and analyzed for water stable isotopes (Oxygen-18 and Deuterium) and chlorofluorocarbons to determine recharge sources and apparent ages.

Our working hypotheses were that: *i*. groundwater in a deep aquifer would have a longer residence time than groundwater in a shallower aquifer, *ii*. contaminants would have a longer residence time in a deep aquifer than in a shallower aquifer, and *iii*. nitrification and denitrification in a deep and pristine aquifer would be slower than in a shallow and contaminated aquifer, and nitrification would be more impacted by contamination than denitrification in the shallower aquifer.

2. Materials and methods

a. Hydrogeological setting

The study area is located within the León-Chinandega plains in the northwestern part of Nicaragua (Fig. 1) and limited to the northeast by the Nicaraguan volcanic chain and to the south west by the Pacific Ocean. The topography of the plains rises smoothly from sea level at the Pacific Ocean towards the volcanic foothills, which extends from 100 to 300 masl The geology of the León-Chinandega plains has three main geological units. The near surface alluvial and quaternary deposits cover most of the surface of the plains. Underlying the quaternary deposits is an older and thicker unit of approximately 220 meters known as Las Sierras Formation (UN, 1974). Las Sierras is composed mainly by pumices and by regular compacted and partially weathered tuffs, which are intercalated by ashes, breccias and volcanic scoria (Kuang, 1971). The third geological unit is the Tamarindo Formation which is defined as a sequence of volcanic ignimbrites and andesitic lavas of tertiary origin (Wilson, 1942). The León-Chinandega aquifer is composed by the quaternary deposits and by Las Sierras Formation. The aquifer thickness ranges from few meters to more than 300 (Corriols

and Dahlin, 2008). The Tamarindo Formation is considered to be the impermeable basement of the aquifer (UN, 1974) . Two different flow systems have been identified in the aquifer. The deep system is recharged in the volcanic chain and later discharged in the central and lower parts of the plains, either as base flow or to pumping wells. The shallow local flow system is recharged in the central and lower parts of the plains and discharged into rivers and pumping wells (Calderón Palma and Bentley, 2007). Isotopic studies suggest that groundwater in the plains is a mixture of waters infiltrated at high elevations and at the plains (Delgado, 2003). Shallow wells are mainly recharged locally and deep wells by the deep flow system (Corriols, 2003).

b. Selected sites

Based on a survey of hydrogeological and anthropogenic conditions (April to May 2005), six of 20 examined wells and springs were selected for bioassays in January to February 2006 (Fig. 1). The selected ground waters represent a gradient of concentrations in OCl pesticides and their metabolites (Appendix, Table 1), that might also differ in provenience and age (shallow and deep aquifers, old and young water). The PP-05 (Cristo Rey) is a drilled well located in the volcanic foothills containing pristine water infiltrated at higher elevations that is used as a community drinking water supply. The PE-01, PE-07 (hand-dug wells) and the MA-02 (spring) are located in the middle part of the plains and represent the pesticide-contaminated aquifers (Dahlberg and Odebjer, 2002). The PE-01 (El Tanque) is located in front of an old pesticides storage place used for mixing and loading of the chemicals for terrestrial and aerial fumigation at a farm where cotton was cultivated in the past, and hence heavily contaminated. The PE-07 is located in the middle of sugar cane fields of an old farm (Hacienda Rancho Grande) where cotton was cultivated in the past, and it is the second most heavily contaminated site. The spring (MA-02, Posolteguilla) is less contaminated than PE-01 and PE-07, and expected to be a true representative of the converging groundwater flow paths of different ages in the aquifer (USGS, 1999). The wells in the plains are assumed to be part of the shallow flow system, mainly supported by local recharge. The other two sites, the PI-01 (Piezometer El Polvón, moderately polluted) and the PE-06 (El Gobierno, pristine) are located in the southern low part of the plains (discharge area). The groundwater was assumed to be a mixture of waters mostly recharged at high elevations (deep flow system).

The assays that required aerobic conditions (nitrification, pp'-DDE degradation, and glyphosate mineralization) were made with groundwater from PP-05, MA-02, and PE-01 that contained more than 2 mg L^{-1} dissolved oxygen concentrations (DO) in most of the sampling occasions (Appendix, Table 2). The sites selected for denitrification assays (PE-06, PI-01, and PE-07) contained less than 2 mg L^{-1} DO. The spring (MA-02) had both high and low DO concentrations and was used for nitrification as well as denitrification assays.

c. Sampling and field parameters

A Grundfos MP1 submersible pump was used to collect water from wells with a depth of less than 90 meters, and a small submersible impeller type pump (Gigant) was used for the shallow wells and the spring. The deepest well PP-05 (> 100 m) was sampled using the installed pump. All wells were purged of at least three casing volumes prior to sampling. Copper tubings were used to collect water for CFC analysis (glass, 125 ml), and Teflon tubing for stable isotopes (Nalgene, 100 ml), nitrification/denitrification assays (plastic gallons), and degradation/ mineralization assays (20 L glass bottles). The CFC sampling was carried out according with the bottle method by the USGS CFC laboratory manual (USGS,

2005). The piezometer was sampled with a peristaltic pump with Teflon tubings given the small diameter of the casing.

The pH and oxidation-reduction potential (ORP) were measured *in situ* with a portable Thermo Orion Model 250Aplus pH meter (Thermo Electron Corporation, Beverly, MA, USA) equipped with combination electrodes with automatic temperature compensation (Appendix, Tables 2 and 3). DO was fixed in the field with $MnSO_4$ solution and alkali-iodide solution in Winkler bottles (~300 ml), protected from sunlight, and titrated in the laboratory (< 12 h) by the azide modification method (4500-O C, (APHA-AWWA-WEF, 1999) (Appendix, Tables 2 and 3). The redox species ($Fe^{2+,}$ Mn^{2+}, H_2S, NO_2^-) were quantified with a portable Hach DR/2010 UV/VIS spectrophotometer (Hach Company, Loveland, CO, USA) using kits for ferrous iron (1,10-phenantroline method), manganese (PAN method), sulfides (methylene blue method), and nitrites (diazotization method) (Appendix, Tables 2 and 3). Electrolytic conductivity was measured using an Orion conductivimeter model 105 (Orion Instruments, London, U.K.) (Appendix, Table 2). Total alkalinity was analyzed by the titration method (2320-B, (APHA-AWWA-WEF, 1999) (Appendix, Table 2). Major element chemical analyses in ground waters were done in the CIRA-UNAN laboratory using standard methods (APHA-AWWA-WEF, 1999) (Appendix, Table 2).

At the beginning of each bioassay, aliquots of groundwater were analyzed for chloride, sulfate and nitrate (ion chromatographic method), total nitrogen (persulfate method + second derivative spectroscopy), ammonia (phenate method), total phosphorus (sulfuric acid-nitric acid digestion + ascorbic acid method) and orthophosphate (ascorbic acid method), according to standard methods (APHA-AWWA-WEF, 1999) (Appendix, Table 3). The total and dissolved organic carbon content (TOC, DOC) was measured following the direct method with a Hach kit after digestion of the samples in a COD (Chemical Oxygen Demand) reactor (Hach Company, Loveland, CO, USA) (Appendix, Table 3).

d. Deuterium, Oxygen 18 analysis, and provenience of water

Deuterium and Oxygen-18 analyses were made by continuous-flow isotope ratio mass spectrometry (CF-IRMS) using an Europa Scientific ANCA-GSL and GEO 20-20 IRMS (PDZ Europa Scientific Instruments, Crewe, U.K.). For Deuterium, a sample volume of 0.4 ml was pipetted into Exetainer tubes and an insert vial containing 5 % platinum on alumina added. The tubes were sealed and then filled with pure hydrogen. Samples were left to equilibrate to allow complete equilibration of the water with the hydrogen gas. After deuterium analysis, the vials were flushed with pure CO_2 and left to equilibrate for Oxygen-18 analysis. Reference waters (including a quality control standard) were prepared in the same manner. The samples were measured against three reference standards of δ^2H (-14.5 ‰, -85.7 ‰, and -155.6 ‰ vs. V-SMOW) and $\delta^{18}O$ (-3.47 ‰, -13.36 ‰, and -23.22 ‰ vs. V-SMOW). All standards are traceable to the primary reference standards of V-SMOW (Vienna -Standard Mean Ocean Water) and SLAP (Standard Light Antarctic Precipitation) distributed by the IAEA (International Atomic Energy Agency, Vienna). Measurement accuracy for triplicate samples was 0.05–0.08 and 0.7–1.9 ‰, respectively, for $\delta^{18}O$ and δ^2H. The study by (Payne and Yurtsever, 1974) was used to calculate the recharge altitude of groundwater with their proposed equation $\delta\ ^{18}O = -5.653 - (0.0026 \times E)$, where E= elevation in meters above mean sea level (masl), and inspired (Dahlberg and Odebjer, 2002) to formulate an equation to calculate the percentage of groundwater infiltrated from altitudes below 280 m and stated as % lowland rain= $100 - 100[(-\delta^{18}O - 5.9)/(7.18-5.9)]$. This is a rough estimation based on Payne and Yurtsever approximation that 75 % of the recharge to the deep part of the aquifer originates from recharge on the higher slopes (> 280 m).

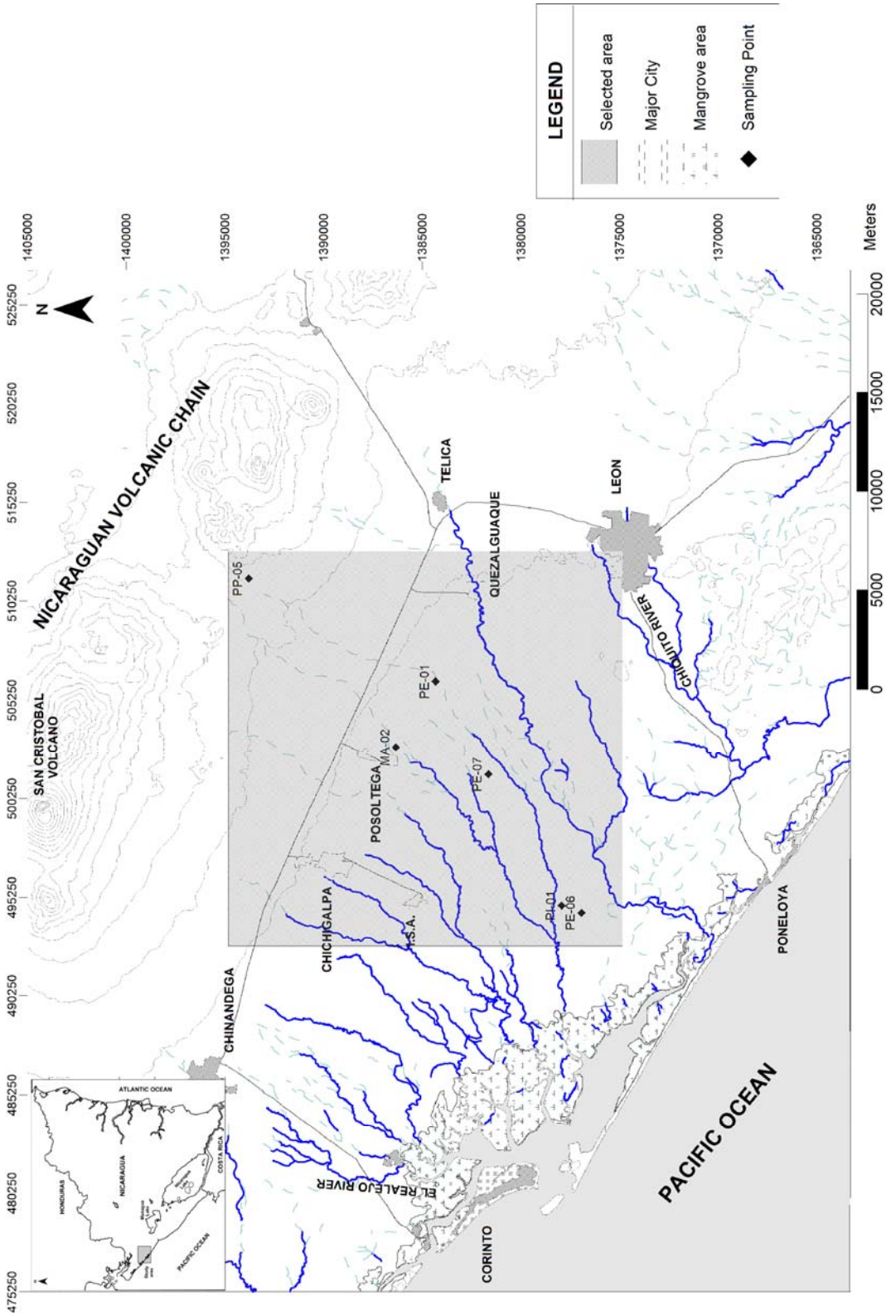

Figure 1. Map of the León-Chinandega study area with the locations of sampling sites

e. Chlorofluorocarbon analysis

Chlorofluorocarbons: CFC-11 (trichlorofluoromethane), CFC-12 (dichloro difluoromethane) and CFC-113 (trichlorotrifluoromethane) were analyzed by gas chromatography (GC) using a purge-and-trap pre-treatment system coupled to a Shimadzu Model GC-8 (Japan) equipped with an electron capture detector (^{63}Ni-ECD) (Busenberg and Plummer, 1992). A calibration curve of six standards was prepared by injecting different volumes of the primary standard Oregon air (R.A. Rasmussen, Oregon Graduate Institute, USA). Given the absence of noble gas measurements (Ar, Ne), the recharge temperature was assumed to be equal to *i*) the estimated average air temperature at the main recharge zone (27 ºC) for all sites, and to *ii*) the temperature measured in the groundwater at sampling (28–34 ºC). The detection limit was 1 pg L^{-1}.

f. Biodegradation of pp'-DDE and mineralization of glyphosate

pp'-DDE (1,1-dichloro-2,2-bis(4'-chlorophenyl) ethylene) is the main metabolite of the OCl pesticide pp'-DDT (1,1,1-trichloro-2,2-bis (4'-chlorophenyl) ethane) that is produced from aerobic biotic degradation, abiotic dehydrochlorination, and even from photochemical decomposition (Thomas et al., 2008). pp'-DDE is the most commonly found OCl compound in ground waters of León-Chinandega (Dahlberg and Odebjer, 2002; Montenegro, 2002; Delgado, 2003; Moncrieff et al., 2008), and has generally been considered as persistent in surface aquatic environments of the area (Carvalho et al., 1999; ATSDR, 2002). The aerobic biodegradation potential of pp'-DDE was calculated from a reduction in the corrected concentration of the compound after accounting for abiotic losses with time (0, 2, 7, 14, and 21 days) in groundwater of the three selected sites. Fifteen replicates of one litre of sample in glass bottles were spiked for each site to obtain ~ 1 ng L^{-1} of pp'-DDE dissolved in methanol (Fisher Chemicals, pesticide grade). Five replicates were abiotic controls and sterilized with formaldehyde (final concentration 1.5 %) at the beginning of the assay, and one replicate was used to analyze ORP, total alkalinity, DO, pH, and conductivity at the end of the assay. The selected pp'-DDE concentration is the guideline value for the protection of aquatic life in freshwater and saltwater ecosystems (ATSDR, 2002) and commonly found in ground waters of the study area (Montenegro, 2002; Moncrieff et al., 2008). The incubation was made at 25 ºC in the dark with the bottles left open for gas exchange but covered with gauze. The samples were stirred manually once a day. The solute extraction was made as described by (Calero et al., 1992), but with a sample volume of 1 L, stirring for 1 hour, and only an acidic destruction that was applied to the 1 ml extracts to eliminate possible interferences and to confirm the identity of pp'-DDE. The concentration of formaldehyde was found adequate to sterilize without interfering with the analysis. The analysis was made using a gas chromatograph Varian 3400 (Varian Inc. Palo Alto, CA, USA) equipped with a ^{63}Ni-ECD and a Varian 8100 autosampler. The GC capillary column was a DB-5 (30 m × 0.25 mm i.d., 0.25 µm film thickness) (J&W Scientific, Tolsom, CA, USA) with hydrogen as carrier gas and nitrogen as make-up gas. The injection port was set to 250 ºC and the detector to 350 ºC while the oven was operated as described by (Calero et al., 1992). Samples and standards (1 µL) were injected in the splitless mode and quantified by the external standard method against a curve of four standards prepared from a stock of pp'-DDE (Chem Service Inc., 99.4 %) dissolved in hexane (Fisher Chemicals, pesticide grade). The half-lives ($t_{½}$) and specific degradation rates (k) were calculated by making semi-logarithmic plots (ln) of remaining substrate concentration versus time of incubation, assuming first-order kinetics (Larsen et al., 2000).

Glyphosate is widely use in the study area (as a herbicide and as a sugar cane ripener) (Montenegro, 2002), and both the parent compound and its main metabolite (amino-methyl phosphoric acid, AMPA) have been found mainly in surface waters but also in ground waters at several locations worldwide, in spite of their expected short half-lives owing to a high potential for microbial degradation (Vereecken, 2005; Borgaard and Gimsing, 2008). The aerobic mineralization potential of glyphosate was calculated from $^{14}CO_2$ production during time-series incubations (1, 3, 5, 10, 15, and 20 days) in spiked groundwater samples (4-6 µg L^{-1} of ^{14}C-glyphosate) from three sites (three replicates per site). The herbicide concentration is higher than normally found in ground waters (< 0.1 µg L^{-1}, (Vereecken, 2005) but required to allow detection of as little as 1 % mineralization in the assay. Nine one hundred ml serum vials were filled up with 60 mL glyphosate-spiked ([phosphono-methyl-^{14}C], American Radiolabeled Chemicals Inc., St. Louis, MO, USA) groundwater and capped with thin rubber septa. The vials were incubated in the dark at 25 ºC on a rotary shaker at 100 rpm. No abiotic control was included since glyphosate degradation is considered to be mainly a biological process mediated by the enzymes C–P lyase or glyphosate oxidoreductase (Borgaard and Gimsing, 2008) and references therein). At sampling, five ml aliquots were withdrawn with a syringe followed by injection of 5 ml of air. The samples were transferred to 20 ml scintillation vials, holding a 6 ml plastic scintillation vial containing 1 ml of 1M KOH and a drop (50 µl) of 10 M NaOH. Immediately following the addition of 1 ml of 2 M HCl to the sample, the 20 ml vial was closed, and the released $^{14}CO_2$ was collected in the KOH solution for 48 hours. The KOH solution was counted for 30 min in a Beckman LS 6500 Multipurpose scintillation counter (Fullerton, CA, USA), after addition of 4 ml of Ultima Gold scintillation cocktail (PerkinElmer Life and Analytical Sciences, Boston, MA, USA). Cumulative $^{14}CO_2$ was corrected for quenching and background radioactivity measured from an incubated blank prepared with distilled water that was treated in the same manner as the samples. First-order rates for ^{14}C-glyphosate degradation were estimated by linear regression of the ln [remaining ^{14}C-glyphosate after subtraction of the cumulative ^{14}C-CO_2 evolved] vs. time of incubation (0-20 days) (Carlsson, 2006). Additionally, doubling times (days) and specific doubling rates (day^{-1}) of $^{14}CO_2$ production were calculated when exponential mineralization (3-20 days) of ^{14}C-glyphosate was observed by making semi-logarithmic plots of cumulated $^{14}CO_2$ production versus time of incubation assuming first-order kinetics (Larsen et al., 2000).

g. Potential nitrification and denitrification assays

Potential nitrification was assayed by adapting the method by (Hart et al., 1994) to water samples. The potential nitrification was calculated as the difference in the ammonia concentration (NH_4-N) between paired samples with one having NH_4-N added and the other not (control), and expressed as µg NH_4-N L^{-1} oxidized per day (Kemp and Dodds, 2002). Three replicates of 250 ml of groundwater per each site were amended with (Payne and Yurtsever, 1974) to obtain ~ 400 µg L^{-1} NH_4-N and incubated together with their controls in Erlenmeyer flasks capped with parafilm on orbital shakers at 175 rpm at 25 ºC and in darkness. Samples for ammonia, nitrite and nitrate analyses were taken daily for up to nine days. Nitrification rates were calculated from the slopes of the linear regression of NH_4 concentrations were plotted versus time of incubation (0-8 and 4-8 days). Linear regression of accumulated nitrite (Pell et al., 1998) and nitrate (Hart et al., 1994) over time (0-9 and 4-9 days), were used to assess the relative sensitivity to pollution of the two phases of nitrification (ammonia oxidation vs. nitrite oxidation), and to determine the main route of disappearance of ammonia. Ammonia was analyzed with the phenate method (4500-NH3 F, (APHA-AWWA-WEF, 1999), and nitrite with the colorimetric method (4500-NO2 B, (APHA-AWWA-WEF, 1999). Both methods were adapted for quantification of small sample

volumes (10 ml) in a UV/VIS spectrophotometer PerkinElmer Lamba 35 (Shelton, CT, USA) with a slit of 1 nm at 543 and 640 nm respectively. Nitrate was analyzed by ion chromatography (Method 4110.B, (APHA-AWWA-WEF, 1999) and quantified in a Dionex DX-100 (Dionex Corporation, Sunnyvale, CA, USA) with a Dionex Ion Pac AS4A (250×4 mm) column and a Dionex Ion Pac AG4A (50×4 mm) guard-column. Samples for nitrate and nitrite analyses were preserved with chloroform (0.2 % v/v) and refrigerated (~ 5 ºC) until analysis (1-2 days) (Hart et al., 1994), while samples for ammonia analysis were acidified (0.1 % v/v) with H_2SO_4 (conc.) and also refrigerated until analysis (< 7 days) (APHA-AWWA-WEF, 1999).

Denitrification was measured using the isotope-pairing technique (Nielsen, 1992) adjusted for water samples, following recommendations by (Kristiansen and Schaanning, 2002). Groundwater samples were flushed with helium for 1 h, supplemented with $Na^{15}NO_3$ (99 %, Cambridge Isotope Lab., Woburn, MA, USA) to produce a final concentration of ~ 3 µM $^{15}NO_3$ and then immediately transferred to 12 ml Exetainer tubes. Five replicates per site and day were incubated statically in the dark at ~ 25 ºC for 2, 5, 12, 16 and 20 days. The time series incubations were terminated by carefully adding 100 µl of formaldehyde (37 %) with a syringe through the septum. Tubes were kept stored upside down at room temperature and in the dark until analysis. The produced N_2 gases ($^{14}N^{14}N$, $^{14}N^{15}N$, $^{15}N^{15}N$) were measured in a PDZ Europa 20-20 continuous flow (CF) IRMS (PDZ Europa Scientific Instruments, Crewe, U.K.) coupled to a GC Agilent/HP G1530A (Hewlett Packard Co., USA) equipped with a head-space autosampler CombiPAL (CTC Analytics AG, Zwingen, Switzerland). Aliquots of 200 µl per replicate of the ~2 ml produced head-space of the Exetainer tubes were injected. The inert gases were separated with a GC column HP-5 (50m × 0.25mm × 0.25µm) kept at 50 ºC. $\delta^{15}N$ values were calculated versus an air reference sample. Calculations of the denitrification rates were made as described in Nielsen (1992), and correcting the concentration of N_2 in the liquid phase using a Bunsen absorption coefficient of 1.45×10^{-2} at 25 ºC (Tiedje, 1994). Indigenous denitrification rates were calculated as D_{14} in (Nielsen, 1992) and potential rates (D_{total}) as the sum of $D_{14}+D_{15}$.

h. Data analysis

Linear regressions were calculated using STATISTICA version 6.1 at a significance level of 5 % (p=0.05) (Stat Soft Inc. 2003, www.statsoft.com). Statistics were made using SPSS for Windows 9.0 (Chicago, IL, USA) at a significance level of 5 % (p=0.05). Data normality, homogeneity of variances, and sphericity were evaluated with Shapiro-Wilk's, Levene's, and Mauchly's tests, respectively. Univariate ANCOVAs of ln–transformed data on remaining pp'-DDE as covariate were used to examine the effects of contamination on degradation. Simple contrasts were conducted to compare sites. The significance of the interaction between site and time was used to assess the difference in regression coefficients (i.e., homogeneity of slopes). Ln-transformed data of ammonia, nitrites, nitrates, remaining [^{14}C-glyphosate] and log cumulated [^{14}C]CO_2 were analyzed with a mixed model of RM-ANOVAs (repeated–measures analysis of variance) within replicates throughout time of incubation and between sites to examine the effects of contamination on nitrification and mineralization rates. Simple contrasts were conducted to compare sites among them, while repeated contrasts were conducted to compare results of each sampling date against the previous.

3. Results and discussion

a. Stable isotopes and provenience of groundwater

The $\delta^{18}O$ and $\delta^{2}H$ signatures varied by 1 ‰ (-6.93 to -5.93) and 5.5 ‰ (-46.42 to -40.82) respectively, along the flow paths, showing an enrichment down gradient from the high altitude recharge area (PP-05) to the low altitude discharge area (Table 4). The $\delta^{18}O$ data shows that the deep drilled well (PP-05) recharged at higher elevations (500 masl) compared to the sites at the plains (350 masl), and the sites at the low altitude discharge area, including the PE-07, seemed to be recharged at lower elevations (110 to 215 masl) (Table 4). Three major sub-zones of recharge can be identified by the isotope data: the high altitude recharge area that received small contribution (20 %) from sources below 280 masl, the plains that received an equal contribution of recharge from high and low altitudes (50 %), and the low altitude discharge area that received the most substantial contribution (75–98 %) from sources below 280 masl (Table 4). Hence, the groundwater from the PP-05 was recharged predominantly at higher altitudes (80 %), and the one from the PE-06 was exclusively recharged at lower elevations (98 %). The groundwater at PE-07, even though situated at the plains seems to be mainly recharged at lower elevations, as the low altitude discharge sites.

Table 4. Stable isotope data ($\delta^{2}H$ and $\delta^{18}O$) data (averages of triplicate measurements) for ground waters from aquifers in León-Chinandega, sampled between April and May 2005, predicted average recharge elevation and percentage of recharge below 280 masl assuming the relations proposed by Payne and Yurtsever (1974).

Site (type of sample, degree of contamination, sub-zone)	$\delta^{2}H_{V\text{-}SMOW}$ (‰)	$\delta^{18}O_{V\text{-}SMOW}$ (‰)	Average recharge altitude (masl)	Percentage recharge < 280 masl
PP-05 (drilled well, pristine, high altitude recharge zone)	-46.42	-6.93	500	20
MA-02 (spring, moderately polluted, plains)	-42.73	-6.53	350	50
PE-01 (dug well, heavily polluted, plains)	-43.66	-6.52	350	50
PE-07 (dug well, heavily polluted, plains)	-42.82	-6.09	175	85
PI-01 (piezometer, moderately polluted, low altitude discharge zone)	-40.84	-6.20	215	75
PE-06 (dug well, pristine, low altitude discharge zone)	-42.62	-5.93	110	98

The isotopic composition and the estimated recharge elevation agrees with that in earlier work (Payne and Yurtsever, 1974); (Dahlberg and Odebjer, 2002; Delgado, 2003). Delgado (2003) noticed that the isotopic composition in precipitation varied monthly, seasonally and annually, whereas groundwater data represented a mix and a long-term average of several years of precipitation events. Delgado (2003) also combined data on stable isotopes in the area from 1974 until 2002 and found a trend of isotopic depletion with

increasing altitude. She also estimated the average recharge elevation for the piezometer (PI-01) to 700 m (17 and 1140 m). The difference probably comes from her averaging of three piezometer nests located at different depths and our single estimation from the deepest nest.

b. Apparent age of waters

Table 5. Chlorofluorocarbon age data for ground waters sampled from aquifers in León-Chinandega between April and May 2005. The apparent age refers to the amount of time elapsed between the apparent recharge date and the collection date. The recharge temperature was assumed to be equal to *i*) the estimated average air temperature at the main recharge zone (27 ºC) for all sites **(in bold)**, and to *ii*) the temperature measured in the ground waters at sampling (28–34 ºC).

Site (type of sample, degree of contamination, sub-zone)	Apparent recharge year			Apparent age (years)			Average apparent age (years)
	CFC-11	CFC-12	CFC-113	CFC-11	CFC-12	CFC-113	
PP-05 (drilled well, pristine, high altitude recharge zone)	**1976.0** 1977.5	**1980.5** 1982.5	**1982.0** 1983.5	**29.0** 27.5	**24.5** 22.5	**23.0** 21.5	**23.8**[a] 22.0[a]
MA-02 (spring, moderately polluted, plains)	**1975.5** 1978.5	**1980.0** 1984.0	**1984.0** 1986.0	**29.5** 26.5	**25.0** 21.0	**21.0** 19.0	**23.0**[a] 20.0[a]
PE-01 (dug well, heavily polluted, plains)	**1975.0** 1977.0	**1977.5** 1981.0	**1985.5** 1987.0	**29.5** 28.0	**27.5** 24.0	**19.5** 18.0	**28.5**[b] 26.0[b]
PE-07 (dug well, heavily polluted, plains)	**1972.5** 1973.5	**1989.0** modern	**C** C	**32.5** 31.5	**16.0** < 5	**C** C	**16.0**[c] < 5[c]
PI-01 (piezometer, moderately polluted, low altitude discharge zone)	**1959.0** 1960.0	**1983.0** 1986.5	**1982.0** 1983.5	**46.0** 45.0	**22.0** 18.5	**23.0** 21.5	**22.5**[a] 20.0[a]
PE-06 (dug well, pristine, low altitude discharge zone)	**1957.0** 1957.5	**1976.0** 1977.0	**C** C	**48.0** 47.5	**29.0** 28.0	**C** C	**29.0**[c] 28.0[c]

C Contaminated because CFC concentration was above 2005 air equilibrium
[a]Average apparent age derived from CFC-12 and CFC-113 ages
[b]Average apparent age derived from CFC-11 and CFC-12 ages
[c]No average age calculated and apparent age relied upon CFC-12 age

Apparent ages of the groundwater samples ranged between 16 and 48 yr, with CFC-11 ages older (2–23.5 yr) compared to the reference CFC-12 ages and the CFC-113 ages generally younger (1.5–8 yr) (Table 5). Two sites, the PE-06 and the PE-07, had CFC-113 concentrations above atmospheric solubilities at the time of sampling and considered as contaminated. After averaging the two most reasonable apparent ages when possible, usually

CFC-12 and 113 derived ages, the range of ages was reduced to between 16 and 29 years. The groundwater from the drilled well was not the oldest (24 yr) but from the two dug wells (~ 29 yr) located at the plains (PE-01) and in the low altitude discharge area (PE-06), and the youngest (16 yr) was from PE-07 at the plains. The same pattern was reproduced when the apparent age was calculated using the *in situ* groundwater temperature at each site, but the ages were 0.5 to 4 years lower due to the temperature effect on the solubility of CFCs, but for the PE-07 that became even younger (< 5 y)

The age pattern may have some potential explanations. First, the higher ages calculated from CFC-11 compared to CFC-12 and CFC-113 may be explained by its higher susceptibility to microbial degradation, although usually linked to anaerobic conditions or DO concentrations < 0.5 mg L^{-1} (Busenberg and Plummer, 1992; Böhlke and Denver, 1995; Cook et al., 1995; Bockgård et al., 2004). In our case, the most important potential degradation of CFC-11 should be at the low altitude discharge sites and at the PE-07 (40–50 %), because of the oxygen-poor conditions (0.4–1.8 mg L^{-1} DO, Appendix Table 3), but some potential CFC-11 degradation may also occur at the recharge site (16 %) and at the plains (7–15 %). The CFC-113 ages when determined, were in good agreement with the CFC-12 ages (5–15 %), except at the PE-01 where they differed by 9 yr.

Second, the groundwater at the drilled pristine well (PP-05) seems young partly because the time required for water to infiltrate through the unsaturated zone (travel time) is not included in the CFC-determined age. That time is usually unimportant for thin unsaturated zones (< 10 m), but significant for thick (Johnston et al., 1998). Information given by pump workers at the site suggests that the thickness of the unsaturated zone at this site was around 100 m. This groundwater was also the most depleted and according to the $\delta^{18}O$ content, it was recharged predominantly at mean elevations at least 150 m higher than the sites located at the plains. Moreover, this groundwater was the only one of the hydrochemical type Mg/Ca–HCO_3 (Appendix Table 2), with high electric conductivity and the highest content of Mg, Na, K and SO_4. Usually, groundwater associated with deeper, older systems has higher electrical conductivity than shallower, younger water because of the longer flow paths and contact times (Focazio et al., 1998). This groundwater also contained relatively low nitrate concentrations (Appendix, Table 2), and no organochlorines were detected (Appendix, Table 1), so we classified it as pristine and as a reference to the impact of agricultural contamination. Groundwater from another drilled well located at 280 masl had a similar $\delta^{18}O$ (-7.0 ‰) as PP-05 (Payne and Yurtsever, 1974) and estimated to be 40 years old in 1970 based on tritium data (Calderón Palma, 2003). If this is the case, we were probably just tracing part of the flow path of this groundwater. We cannot exclude the possibility that the deep groundwater was mixed with young groundwater or air enriched in CFCs during pumping, since this was the only site that was not sampled with peristaltic pumps adjusted to discharge low flows to disturb groundwater samples as little as possible.

Third, the youngest apparent age calculated for the PE-07 may be due to a high turn over of water, since the well is located in the middle of irrigated sugar cane fields. This would also explain the odd isotopic signature compared to the other sites at the plains, including a relatively high contribution of waters recharged at lower elevations. The hydrochemical data also places it together with the other sites at the plains, with low electric conductivity and similar ion concentrations (chloride, sulfate, sodium) (Appendix, Table 2). This high turnover may also explain the reduction and even disappearance of some OCl contaminants in the past few years when the data in Dahlberg and Odebjer (2002) are compared with ours in 2005 (Appendix, Table 1). For example, we did not detect any

toxaphene that was the main OCl contaminant (117 ng L^{-1}) at the site in 2002, and the concentrations of β-BHC, endrin, and dieldrin were lower and even below detection limits for aldrin.

Accurate groundwater ages determined from CFC content are usually only possible to obtain for the limiting case of piston flow and for water recharged in the period 1955 to 1995 (Busenberg and Plummer, 1992). In our case we have a heterogeneous system with a significant mixing of waters of different proveniences (^{18}O data) and probably of different ages, hence these CFC derived ages should be considered as minimum ages. Additional environmental tracers (e.g., tritium) should be useful to discriminate between potential sources of error in the CFC derived ages.

c. pp'-DDE biodegradation and glyphosate mineralization

All samples degraded pp'-DDE at high rates (Table 6). Data sets of pp'-DDE concentrations presented significant differences when the polluted sites were compared to the reference pristine site (p=0.000), and between each other (p=0.000). This was expected since the initial concentrations of pp'-DDE in the raw ground waters were different due to the gradient of *in situ* concentrations (from pristine to heavily polluted). The degradation rates were (10–20 %) higher at the contaminated sites (MA-02 and PE-01) than at the pristine site (PE-05), but the differences were insignificant (p=0.287–0.733).

The ^{14}C-glyphosate was mineralized at low rates in all three waters (Table 6). The potential degradation of ^{14}C-glyphosate was significantly different in samples from the polluted sites compared to the reference pristine site (p=0.000–0.001), and significantly different to each other (p=0.000). The slopes of the regression lines in the polluted sites were significantly different to the one of the reference site (p=0.000–0.040), indicating significantly higher degradation rates (20–65 %) and concomitant lower half-lives (90–200 days) for MA-02 and PE-01, respectively.

The doubling times (Dt, days) and doubling rates (day^{-1}) for $^{14}CO_2$ production from metabolism of ^{14}C-glyphosate were 9 and 8×10^{-2} for the pristine site, 8 and 9×10^{-2} for the moderately polluted spring, and 7 and 10×10^{-2} for the heavily polluted sites, respectively. The added ^{14}C-glyphosate was mineralized significantly (p=0.000) from the first day of incubation (0.2—0.6 %) until achieving 4–6 % cumulated $^{14}CO_2$ evolution after 20 days. The polluted sites (MA-02 and PP-01) had higher significant CO_2 accumulation (p=0.000–0.024) than the reference pristine site (PP-05) and were significantly different from each other (p=0.000). The slopes of the regression lines of log [cumulated $^{14}CO_2$ produced] data (doubling rates) in the polluted sites were significantly different to the one of the reference site (p=0.000–0.002), indicating higher specific doubling rates (15–25 %) and concomitant lower doublings time (1.1–1.7 days) for MA-02 and PE-01, respectively.

Table 6. Average potential first order rate constants (*k*), half-lives (t½), correlation coefficients and significance of linear regressions for aerobic pp'-DDE biodegradation and ^{14}C-glyphosate mineralization in ground waters from León-Chinandega. The biodegradation rates were calculated from the slopes of the regression lines in plots of ln [remaining pp'-DDE after correction for abiotic degradation] *versus* time of incubation (0-21 days). The water was spiked with 1 ng L^{-1} of pp'-DDE and sets of two replicates per sampling day and site were sacrificed five times during the incubation, along with an abiotic control. The mineralization rates were calculated from the slopes of the regression lines in plots of ln [^{14}C-glyphosate remaining after subtracting the cumulative ^{14}C-CO_2 evolved] *versus* time of incubation (0-20 days). The ground waters were initially spiked with 4-6 µg L^{-1} of ^{14}C-glyphosate in sets of three replicates that were sampled seven times during the incubation.

Site (type of sample, degree of pollution)	pp'-DDE		^{14}C-glyphosate	
	k (day^{-1})	t½ (days)	*k* (day^{-1})	t½ (days)
PP-05 (drilled well, pristine)	0.0219 (r^2=0.8826)*	32	0.0014 (r^2=0.9798)*	495
MA-02 (spring, moderately polluted)	0.0239 (r^2=0.8713)*	29	0.0017 (r^2=0.9931)*	408
PE-01 (dug well, heavily polluted)	0.0279 (r^2=0.9159)*	25	0.0023 (r^2=0.9904)*	301

pp'-DDE is considered persistent in surface aquatic environments of the study area (Carvalho et al., 1999) as in other tropical or subtropical environments (Carvalho et al., 2002) because of the relatively low contribution of its dechlorination metabolite DDMU (1-chloro-2,2-bis(4'-chlorophenyl)ethane) to the DDT pool. The fast degradation in our assays may come from the release of pp'-DDE from the low bioavailability under field conditions due to sorption to aquifer material (Van Straalen and Løkke, 1997; Washington, 2001). In bioassays, chemicals are usually added freshly to the substrate, while in the field, equilibrium is established over a long period of time, and extrapolation is only valid only when the bioavailable concentration is constant (Van Straalen and Løkke, 1997). However, rate estimates with groundwater samples without aquifer material may be useful to determine the potential for contaminant degradation and potential differences in degradation at different sites, and whether microbial populations are better adapted for contaminant degradation at contaminated sites than at uncontaminated (Anderson, 1997 and references therein).

The degradation rates were much lower and the half-lives much longer when the the disappearance of glyphosate was calculated from mineralization (CO_2 evolution) than from disappearance of the parent compound in river and ground waters (t½=2.5–32 d) (Goldsborough and Brown, 1993; Mallat and Barceló, 1998). The difference is methodological dependent, and Fomsgaard (1995) recommended that both CO_2 evolution and disappearance of the parent compound are measured. As an example, Helweg (1993) found that half of the herbicide mecoprop had disappeared but only 12 % was mineralized.

Evolution of CO_2 expresses the ultimate or total biodegradation, which is supposed to be the "real degradation", and is the basis of the regulatory requirements on biodegradability of industrial chemicals (OECD, 2006). But 100 % of a compound may not necessarily be mineralized since some metabolites of degradation may be used directly for cell growth (De Henau, 1993), especially in a nutritious compound as glyphosate. Moreover, AMPA is the most commonly detected metabolite over sarcosine in soils, probably because the former is strongly sorbed through the phosphonate group and protected against microbial degradation (Borgaard and Gimsing, 2008 and references therein).

Independent of the methodological problems detected in the bioassays, the patterns of the potential biodegradation rates were the same, and the most heavily polluted site had the highest potential, the moderately polluted an intermediate capacity and the reference pristine site, the lowest. This implies that the shallow ground waters (< 10 m) had higher microbial activity or higher capacity to use the pesticides than the groundwater from the pristine deep aquifer (~100 m). In general, the microbial activity is expected to be higher in shallow aquifers compared to deep pristine aquifers, and that degradation occurs more readily at sites previously exposed to the substrate due to adaptation (Cavalier et al., 1991; Anderson, 1997). This higher capacity to degrade glyphosate in polluted sites was also observed by Kools et al. (2005) who introduced the idea of using the degradation rate as an indicator of ecosystem health to test the effects of heavy metal pollution on soils. Aamand et al. (1989) used the length of the lag phase to show that the bacterial community at a heavily polluted well had a higher degree of adaptation to hydrocarbon degradation than the community from a less polluted.

d. Potential nitrification and denitrification

The rates of ammonia disappearance were 10 to 80 % higher at the polluted sites than at the reference site (PP-05) but significantly different (p=0.001–0.048) only during the last five days of incubation and decreased repeatedly and significantly with incubation time (p=0.000–0.041) (Table 7). The highest, significantly different disappearance rates (p=0.024–0.036) were measured at the moderately polluted spring and the lowest at the heavily polluted dug well (p=0.666–0.693). However, the polluted sites were significantly different to each other (p=0.014–0.020). The ammonia disappearance at the spring was characterized by a biphasic pattern with a first phase (0–4 days) of low rate (13 µg NH_4-N L^{-1} d^{-1}) (data not presented) and a second phase (4–8 days) of high rate (98 µg NH_4-N L^{-1} d^{-1}).

The potential ammonia oxidation rates were significantly (p=0.018) higher (28–30×) at the moderately polluted spring site (MA-02) than at the pristine reference site (PP-05) (Table 7). The heavily polluted had the lowest rate (40 %) (p=0.378). The polluted sites had significantly different oxidation rates (p=0.006). Nitrites production started to be significant after three days of incubation (p=0.000–0.008). All sites had four days lag phases (data not presented).

Table 7. Potential nitrification rates, ammonia oxidation rates, nitrites oxidation rates, correlation coefficients and significance of linear regressions of ground waters initially amended with 400 μg L^{-1} of NH_4-N. The rates were calculated from the slopes of the regression lines in plots of NH_4-N disappearance (nitrification), NO_2-N production (ammonia oxidation), or NO_3-N production (nitrites oxidation) versus time of incubation (days) of data partitioned into two periods. Average of three replicates per site is shown.

Site (type of sample, degree of pollution)	Nitrification rates (μg NH_4-N L^{-1} d^{-1})	Ammonia oxidation rates (μg NO_2-N L^{-1} d^{-1})	Nitrites oxidation rates (μg NO_3-N L^{-1} d^{-1})
PP-05 (drilled well, pristine)	39 (r^2=0.7967)* (0-8 d) 54 (r^2=0.6361)* (4-8 d)	0.9 (r^2=0.6158)* (0-8 d) 1.9 (r^2=0.7045)* (4-8 d)	13 (r^2=0.3480)* (0-9 d) 28 (r^2=0.4858)* (4-9 d)
MA-02 (spring, moderately polluted)	50 (r^2=0.7888)* (0-8 d) 98 (r^2=0.9287)* (4-8 d)	28 (r^2=0.7127)* (0-8 d) 56 (r^2=0.9004)* (4-8 d)	13 (r^2=0.0926) (0-9 d) 34 (r^2=0.1521) (4-9 d)
PE-01 (dug well, heavily polluted)	35 (r^2=0.6909)* (0-8 d) 49 (r^2=0.4613)* (4-8 d)	0.6 (r^2=0.3218)* (0-8 d) 1.1 (r^2=0.2745)* (4-8 d)	19 (r^2=0.1907)* (0-9 d) 22 (r^2=0.0669) (4-9 d)

The potential nitrites oxidation rates were not significantly different between the sites (p=0.432–0.842), and significant nitrate production was only detected in the last two days of incubation (p=0.020–0.047). This lack of statistical significance in the rates could be partially explained by the very poor temporal correlation of NO_3 production data, especially for the two polluted sites (see correlation coefficients) (Table 7). They had relatively high measurement uncertainties (~ 155 μg L^{-1}, calculated as suggested in NMKL 1997) associated with their relatively high initial nitrate concentrations (Appendix, Table 2) that accounted for ~ 40 % of the total expected NO_3-N production (400 μg L^{-1}). Higher initial NH_4-N concentrations (> 1 mg L^{-1}) would have been needed to detect differences in nitrite oxidation between sites. The reference site had a relatively low analytical uncertainty for the nitrate data (0.01 μg L^{-1}), but still a poorer regression than ammonia disappearance and nitrite production.

The pattern for nitrogen nitrified via the two phases (ammonia vs. nitrites oxidation) it was different between the sites. For example, a small percentage (< 2 %) seemed to be nitrified via ammonia oxidation (see rates) in the PP-05 and the PE-01, while about 55 % was nitrified at the spring. Hence, most probably the bulk of the disappeared ammonia (> 70 %) in the former sites was directly nitrified to nitrates.

Table 8. Average indigenous (D_{14}), and potential (D_{total}) denitrification rates in nM N d^{-1} and standard deviation measured with the isotope pairing technique after two days of incubation in groundwater samples from the study area. The samples were incubated in the dark and initially supplemented with 3 µM $^{15}NO_3$. The rates were calculated as described in Nielsen (1992) and average of four replicates per site is shown.

Site (type of sample, degree of pollution)	*In situ* $^{14}NO_3$ (µM)	Denitrification rates (nM N day^{-1})		
		D_{14}	D_{15}	D_{total}
PE-06 (dug well, pristine)	103	4.6 ± 0.4	4.0 ± 0.1	8.6 ±0.5
MA-02 (spring, moderately polluted)	444	2.8 ± 0.2	3.1 ± 0.1	6.0 ± 0.2
PI-01 (piezometer, moderately polluted)	95	6.4 ± 1.1	5.3 ± 0.7	11.7 ± 1.8
PE-07 (dug well, heavily polluted)	71	4.2 ± 0.4	4.1 ± 0.6	8.3 ± 0.9

The indigenous and potential denitrification were very low for all sites but higher at the moderately polluted piezometer (40 %), intermediate at the heavily polluted site (<10 %), and lower at the moderately polluted spring (60 %) than at the pristine reference site (Table 8). The samples were incubated in time series ranging from two to twenty days, because we expected a lag phase and low rates as is commonly seen in water column experiments (Kristiansen and Schaanning 2002). But when data on $^{14}N^{15}N$ excess versus time of incubation were plotted, we realized that all data sets had an increase at the beginning of the assays and then a drop so the indigenous and potential denitrification rates may be underestimated and only the higher calculated rates are presented (after two days of incubation). The spring (MA-02) that is not located in the discharge area, but sometimes presented low oxygen conditions, was tested just for comparison of denitrifying activity between the plains and the discharge area.

It is not possible to compare rates of nitrification and denitrification in other studies because they are usually given in units per area. For both processes, groundwater from the moderately polluted sites MA-02 (nitrification) and PI-01 (denitrification) had the highest rates, and the heavily polluted sites PE-01 (nitrification) and PE-07 (denitrification) had the lowest rates. The three sites located at the discharge area presented higher denitrifying activity than the spring (MA-02) which coincides with lower *in situ* NO_3 concentrations in the former sites. Denitrification was less affected by pollution than nitrification, probably because the most polluted site in the denitrification bioassays (PE-07) was less polluted than the most contaminated in the nitrification bioassays (PE-01) which was the only site polluted with toxaphene (Appendix, Table 1). Toxaphene is known to inhibit ammonia oxidation (>25 %) at 12× lower application rates (kg/ha) than dieldrin, DDT and endrin that were the other OCls at both sites (Stevenson, 1982). Delgado (2003) noted a decrease in nitrate levels with depth in the piezometer (PI-01), and a correlation between inorganic nitrogen and redox

measurements (Eh and DO) indicating *in situ* denitrification, which agrees with our finding of the highest indigenous and potential rates at the site.

4. Conclusions

I. Both the pristine deep aquifer and especially the shallow polluted aquifer have degradation potential for DDE and glyphosate, and the contaminated site may have strains adapted for faster degradation.

II. Moderate pollution did not inhibit nitrification and denitrification.

III. The most contaminated groundwater inhibited nitrification, most probably due to the presence of toxaphene.

IV. Degradation of CFC-11 was detected mainly in the discharge area and most likely due to poor oxygen conditions, but some was also detected in the plains.

V. The isotopic composition of groundwater has a relatively low temporal variation when compared with data from other studies, and there was a trend of isotopic enrichment with decreasing altitude.

VI. The persistence of the *in situ* pollutants is not explained by the apparent age of water or the provenience.

VII. A combination of age tracers should be used to resolve the age distribution in the area due to the complexity of flow paths and mixing of waters.

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Appendix: Tables

Table 1. Organochlorine pesticides and metabolites concentrations (ng L^{-1}) found at heavily (PE-01, PE-07) and moderately (MA-02, PI-01) polluted sites, type of groundwater, and localization in sub-zones of the study area. PP-05 (drilled well, recharge) and PE-06 (dug well, discharge) were analyzed but no organochlorines were detected and hence the ground waters were assumed as pristine.

Site/Pesticide or metabolite	PE-01 (dug well, plains)	MA-02 (spring, plains)	PE-07 (dug well, plains)	PI-01 (piezometer, discharge)
Toxaphene	145[a] 283–329[c]		117[a]	77[b]
ß-BHC	75[a]		30[a] 2.9[c]	
Endrin	11.2[a] 8.2–9.3[c]		23[a] 2.1[c]	
pp'-DDT	 7.4–9.1[c]	 9.6[c]	2.6[a] 15.6[c]	
pp'-DDD	11.4[a] 6.6–7.1[c]	 1.5–2.8[c]	6.9[a] 2.5[c]	4.1[b]
Dieldrin	4.4[a] 3.1[c]	 1.5[c]	4.2[a] 0.6[c]	5.9[b] 6.6[c]
Aldrin	0.9[a] 1.6[c]		0.6[a]	
pp'-DDE	2.5[a] 2.1–2.4[c]	 1.0–1.3[c]	1.5[a] 1.2[c]	1.5[b] 0.2[c]

[a]Dahlberg and Odebjer (2002) [b]Delgado (2003) [c]This study (2005)

Table 2. Hydrochemical type and ranges in ambient values and concentrations of important physico-chemical parameters in ground waters of the six study sites.

Site/type Parameters	PP-05 Mg/Ca HCO_3 (recharge)	MA-02 Ca HCO_3 (plains)	PE-01 Ca/Mg HCO_3 (plains)	PE-07 Ca/Mg HCO_3 (plains)	PI-01 Ca /HCO_3 (discharge)	PE-06 Ca/Mg HCO_3 (discharge)
pH	6.50–6.98	6.52–6.80	6.48–6.87	6.70–6.89	7.16–7.50	6.66–6.77
Temperature (º C)	28–30	33–34	30–32	29–31	31–32	30
Dissolved oxygen (mg L^{-1})	4.7–5.0	<0.2–5.8	5.1–6.2	1.0–1.8	0.6–0.8	0.4–1.0
Electrical conductivity (µs cm^{-1})	745–766	409–465	315–414	434–450	750–768	638–735
Calcium (mg L^{-1})	54	40	27	40	100	54
Magnesium (mg L^{-1})	39	14	11	21	26	24
Total alkalinity (mg L^{-1})	291–302	146–152	102–110	185	317	199
Sodium (mg L^{-1})	45	28	14	18	21	37
Potassium (mg L^{-1})	14	9	9	5	7	4.5
Dissolved silica SiO_2 (mg L^{-1})	95	104	96	79	88	71
Chloride (mg L^{-1})	30–31	14	12–16	13	50	54
Sulfate (mg L^{-1})	74	26	18–19	29	42	54
NO_3-N (mg L^{-1})	1.2–1.8	3.7–6.4	6.3–9.9	1.0–1.8	1.3–1.4	1.3–1.4

Table 3. Ranges in ambient values and concentrations of redox species and nutrients in ground waters of the six study sites.

Site/ Parameters	PP-05 (recharge)	MA-02 (plains)	PE-01 (plains)	PE-07 (plains)	PI-01 (discharge)	PE-06 (discharge)
ORP (mV)	207–371	220–228	207–210	234	232	228
Dissolved oxygen (mg L^{-1})	4.7–5.0	<0.2–5.8	5.1–6.2	1.0–1.8	0.6–0.8	0.4–1.0
Dissolved organic carbon (DOC) (mg L^{-1})	0.3–2.1	<0.3–1.0	0.5–2.1	<0.3–2.4	0.3–3.2	<0.3–2.4
Total nitrogen (mg L^{-1})	1.4–1.8	6.2–6.7	6.5–10.2	1.0–1.8	1.4	1.5
NO_3-N (mg L^{-1})	1.2–1.8	3.7–6.4	6.3–9.9	1.0–1.8	1.3–1.4	1.3–1.4
NH_4-N (µg L^{-1})	8–140	6–180	6–130	2–40	8–40	5–60
NO_2-N (µg L^{-1})	4–8	4–11	9	ND	5–9	5–6
Ortophosphate PO_4-P (µg L^{-1})	100–103	87–154	91–96	22	45	25
Fe^{2+} (µg L^{-1})	ND–40	ND–90	ND–10	10–40	ND	10
Mn^{2+} (µg L^{-1})	34–38	26–34	20–33	42–43	57–120	81–281

6. References

Aamand, J., Jørgensen, C., Arvin, E., and Jensen, B. K., 1989. Microbial adaptation to degradation of hydrocarbons in polluted and unpolluted groundwater. J. Contam. Hydrol. 4, 299-312.

Alexander, M., 1994. Biodegradation and bioremediation. Academic Press Inc., London.

Anderson, T. A., 1997. Ecology and biogeochemistry of *in situ* groundwater bioremediation. In Jones (Eds.), Advances in microbial ecology, Plenum Press, New York.

APHA-AWWA-WEF, 1999. Standard methods for the examination of water and wastewater. 20th Edition. American Public Health Association, American Water Works Association, Water Environment Federation.

ATSDR (2002). Toxicological profile for DDT, DDE and DDD. U. S. Agency for Toxic Substances and Disease Register.

Bengtsson, G., and Bergwall, C., 1995. Heterotrophic denitrification potential as an adaptive response in groundwater bacteria. FEMS Microbiol. Ecol. 16, 307-318.

Bockgård, N., Rodhe, A., and Olsson, K. A., 2004. Accuracy of CFC groundwater dating in a crystaline bedrock aquifer: data from a site in sothern Sweden. Hydrogeol. J. 12, 171-183.

Böhlke, J.-K., 2002. Groundwater recharge and agricultural contamination. Hydrogeol. J. 10,153-179.

Böhlke, J. K., and Denver, J. M., 1995. Combined use of groundwater dating, chemical, and isotopic analyses to resolve the history and fate of nitrate contamination in two agricultural watershed, Atlantic coastal plain, Maryland. Water Resour. Res. 31, 2319-2339.

Borgaard, O. K., and Gimsing, A. L., 2008. Fate of glyphosate in soil and the possibility of leaching to ground and surface waters: a review. Pest Manag. Sci. 64, 441-456.

Busenberg, E., and Plummer, N., 1992. Use of chlorofluorocarbons (CCl_3F and CCl_2) as hydrologic tracers and age-dating tools: the alluvium and terrace system of Central Oklahoma. Water Resour. Res. 28, 2257-2283.

Calderón Palma, H., 2003. Numerical modeling of the groundwater flow system in a sub- basin of the León-Chinandega aquifer, Nicaragua. University of Calgary, Canada.

Calderón Palma, H., and Bentley, L. R., 2007. A regional-scale groundwater flow model for the Leon-Chinandega aquifer, Nicaragua. Hydrogeol. J. 15, 1457-1472.

Calero, S., Fomsgaard, I. S., Lacayo, M. L., Martínez, V., and Rugama, R., 1992. Preliminary study of 15 organochlorine pesticides in Lake Xolotlán, Nicaragua. Chemosphere 24, 1413-1419.

Carlsson, C., 2006. Limitations and possibilities for microbial degradation of organic contaminants in aquifers. PhD thesis, Lund University, Sweden.

Carvalho, F. P., González-Farías, F., Villeneuve, J.-P., Cattini, C., Hernández-Garza, M., Mee, L. D., and Fowler, S. W., 2002. Distribution, fate and effects of pesticide residues in tropical coastal lagoons of Northwestern Mexico. Environ. Technol. 23, 1257-1270.

Carvalho, F. P., Montenegro-Guillén, S., Villeneuve, J.-P., Cattini, C., Bartocci, J., Lacayo, M., and Cruz, A., 1999. Chlorinated hydrocarbons in coastal lagoons of the pacific Coast of Nicaragua. Arch. Environ. Contam. Toxicol. 36, 132-139.

Cavalier, T. C., Lavy, T. L., and Mattice, J. D., 1991. Persistence of selected pesticides in ground-water samples. Ground water 29, 225-231.

Cook, P. G., Solomon, D. K., Plummer, L. N., Busenberg, E., and Schiff, S. L., 1995. Chlorofluorocarbons as tracers of groundwater transport processes in a shallow, silty sand aquifer. Water Resour. Res. 31, 425-434.

Corriols, M., 2003. Hydrogeological, geophysical and hydrochemical investigations in the León-Chinandega plains, Nicaragua. Licenciate thesis, Lund Institute of Technology, Sweden.

Corriols, M., and Dahlin, T., 2008. Geophysical characterization of the León-Chinandega aquifer, Nicaragua. Hydrogeol. J. 16, 349-362.

Dahlberg, C., and Odebjer, W., 2002. Investigation of hydrochemical characteristics and pesticide
concentrations in groundwater at Posoltega, León-Chinandega plains, Nicaragua. MSc. thesis, Lund Institute of Technology, Sweden.

De Henau, H., 1993. Biodegradation. In Calow, P. (Eds.), Handbook of ecotoxicology, University Press, Cambridge.

Delgado, V., 2003. Groundwater in a coastal plain, Nicaragua. MSc. thesis, University of Calgary, Canada.

Domsch, K. H., Jagnow, G., and Anderson, T.-H., 1983. An ecological concept for the assessment of side-effects of agrochemicals on soil microorganisms. Res. Rev. 68, 66-105.

Focazio, M. J., Plummer, L. N., Böhlke, J.-K., Busenberg, E., Bachman, L. J., and Powars, D. S. (1998). Preliminary estimates of residence times a apparent ages of ground water in the Chesapeake Bay Watershed, and water quality data from a survey of springs. United States Geological Survey, Report 07-4225.

Fomsgaard, I. S., 1995. Degradation of pesticides in subsurface soils, unsaturated zone- a review of methods and results. Intern. J. Environ. Anal. Chem. 58, 231-245.

Goldsborough, L. G., and Brown, D. J., 1993. Dissipation of glyphosate and aminomethylphosphonic acid in water and sediments of boreal forest ponds. Environ. Toxicol. Chem. 12, 1139-1147.

Hart, S. C., Stark, J. M., Davidson, E. A., and Firestone, M. K., 1994. Nitrogen mineralization, immobilization, and nitrification. In Weaver, R. W., Angle, S., Bottomley, P., Bezdicek, D.,

Smith, S., Tabatabai, A. and Wollum, A. (Eds.), Methods of soil analysis. Part 2: Microbiological and biochemical properties, Soil Science Society of America, Inc.

Helweg, A., 1993. Degradation and adsorption of ^{14}C-mecoprop (MCPP) in surface soils and in subsoil. Influence of temperature, moisture content, sterilization and concentration on degradation. Sci. Total Environ. 132, 229-241.

Johnston, C. T., Cook, P. G., Frape, S. K., Plummer, L. N., Busenberg, E., and Blackport, R. J., 1998. Ground water age and nitrate distribution within a glacial aquifer beneath a thick unsaturated zone. Ground water 36, 171-180.

Kemp, M. J., and Dodds, W. K., 2002. Comparisons of nitrification and denitrification in prairie and agriculturally influenced streams. Ecol. Applic. 12, 998-1009.

Kools, S. A. E., Van Roovert, M., Van Gestel, C. A. M., and Van Straalen, N. M., 2005. Glyphosate degradation as a soil health indicator for heavy metal polluted soils. Soil Biol. Biochem. 37, 1303-1307.

Kristiansen, S., and Schaanning, M. T., 2002. Denitrification in the water column of an intermittently anoxic fjord. Hydrobiologia 469, 77-86.

Kuang, J. (1971). Geology of the Pacific coast of Nicaragua. Report No. 3 (in spanish). Geographical Survey of Nicaragua, Managua.

Lapworth, D. J., and Goody, D. C., 2006. Source and persistence of pesticides in a semi- confined chalk aquifer of southeast England. Environ. Pollut. 144, 1031-1044.

Larsen, L., Sorensen, S. R., and Aamand, J., 2000. Mecoprop, isoproturon, and atrazine in and above a sandy aquifer: vertical distribution of mineralization potential. Environ. Sci. Technol. 34, 2426-2430.

Mallat, E., and Barceló, D., 1998. Analysis and degradation study of glyphosate and of aminomethylphosphonic acid in natural waters by means of polymeric and ion- exchange solid-phase extraction columns followed by ion chromatography-post-colum derivatization with fluorescence detection. J. Chromatogr. A 823, 129-136.

Moncrieff, J. E., Bentley, L. R., and Calderón Palma, H., 2008. Investigating pesticide transport in the León-Chinandega aquifer, Nicaragua. Hydrogeol. J. 16, 183-197.

Montenegro, K., 2002. Ecological implications of DDT and glyphosate - linking toxicology and trophic cascading. Licentiate thesis, Lund University, Lund.

Nielsen, L. P., 1992. Denitrification in sediment determined from nitrogen isotope pairing. FEMS Microbiol. Ecol. 86, 357-362.

OECD (2006). Part 1: Principles and strategies related to the testing of degradation of organic chemicals, p. 17. Organization for Economic Co-operation and Development.

Osenbrück, K., Fiedler, S., Knöller, K., Weise, S. M., and Sültenfuß, 2006. Timescales and development of groundwater pollution by nitrate in drinkin water wells of the Jahna-Aue, Saxonia, Germany. Water Resour. Res. 42, W12416.

Pauer, J. J., and Auer, M. T., 2000. Nitrification in the water column and sediment of a hypereutrophic lake and adjoining river system. Wat. Res. 34, 1247-1254.

Payne, B. R., and Yurtsever, Y. (1974). Environmental isotopes as a hydrogeological tool in Nicaragua. In Isotope techniques in groundwater hydrology. International Atomic Energy Agency, Vienna, Austria.

Pell, M., Stenberg, B., and Torstensson, L., 1998. Potential denitrification and nitrification tests for evaluation of pesticide effects in soil. Ambio 27, 24-28.

Solomon, D. K., Cook, P. G., and Sanford, W. E., 1998. Dissolved gases in subsurface hydrology. In Kendall, C. and MacDonnell, J. J. (Eds.), Isotope tracers in catchment hydrology, Elsevier Science B. V., Amsterdam, pp. 291-318.

Stevenson, F. J., 1982. Nitrogen in agricultural soils. American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc., Madison.

Taylor, R. G., Cronin, A. A., Lerner, D. N., Tellam, J. H., Bottrell, S. H., Rueedi, J., and Barret, M. H., 2006. Hydrochemical evidence of the depth of penetration of anthropogenic recharge in sandstone aquifers underlying two mature cities in the UK. Appl. Geochem. 21, 1570-1592.

Thayalakumaran, T., Bristow, K. L., Charlesworth, P. B., and Fass, T., 2008. Geochemical conditions in groundwater systems: implications for the attenuation of agricultural nitrate. Agric. Water Manage. 95, 103-115.

Thomas, J. E., Ou, L.-T., and Al-Agely, A. A., 2008. DDE remediation and degradation. In Whitacre, D. M. (Eds.), Reviews of environmental contamination and toxicology, pp. 55-69.

Tiedje, J. M., 1994. Denitrifiers. In Weaver, R. W., Angle, S., Bottomley, P., Bezdicek, D., Smith, S., Tabatabai, A. and Wollum, A. (Eds.), Methods of soil analysis. Part 2: Microbiological and biochemical properties, Soil Science Society of America, Inc.

UN (1974). A study of groundwater in the Pacific coast region, Volume I., Chinandega. United Nations, New York.

USGS, 1999. Discharge, nitrate load, and residence time of ground water in the Chesapeake Bay Watershed. U.S. Geological Survey, FS-150-99.

USGS, 2005. CFC sampling method-bottles. U. S. Geological Survey. http://water.usgs.gov/lab/chlorofluorocarbons/sampling/bottles/

Van Beelen, P., and Doelman, P., 1997. Significance and application of microbial toxicity tests in assessing ecotoxicological risks of contaminants in soil and sediment. Chemosphere 34, 455-499.

Van Straalen, N. M., and Løkke, H., 1997. Scientific basis for extrapolating results from soil ecotoxicity tests to field conditions and the use of bioassays. In Van Straalen, N. M. and Løkke, H. (Eds.), Ecotoxicological risk assessment of contaminants in soil, Chapman & Hall, London, pp. 25-50.

Vereecken, H., 2005. Mobility and leaching of glyphosate: a review. Pest Manag. Sci. 61, 1139-1151.

Washington, J. W., and Cameron B. A., 2001. Evaluating degradation rates of chlorinated organics in groundwater using analytical models. Environ. Toxicol. Chem. 20, 1909-1915.

Wilson, T. C. (1942). Geology of the Pacific coast area, Nicaragua. Summary report. Geological Survey of Nicaragua, Managua.

The following is a list of Doctoral theses from the Section of Chemical Ecology and Ecotoxicology, Department of Ecology, University of Lund, Sweden

1. ANDERS TUNLID, Chemical signatures in studies of bacterial communities. Highly sensitive analyses by gas chromatography and mass spectrometry. October 3, 1986

2. ANDERS THURÉN, Phthalate esters in the environment: analytical methods, occurrence, distribution and biological effects. November 4, 1988.

3. PETER SUNDIN, Plant root exudates in interactions between plants and soil microorganisms. A gnobiotic approach. March 16, 1990.

4. ANDERS VALEUR, Utilization of chromatography and mass spectrometry for the estimation of microbial dynamics. October 16, 1992.

5. HANS EK, Nitrogen acquisition, transport and metabolisation in intact ectomycorrhizial assocoations studied by 15N stable isotope techniques. May 14, 1993

6. ROLAND LINDQUIST, Dispersal of bacteria in ground water-mechanism, kinetics and consequences for facilitated transport. December 3, 1993.

7. ALMUT GERHARDT, Effect of metals on stream invertebrates. February 17, 1995.

8. OLOF REGNELL, Methyl mercury in lakes: factors affecting its production and partitioning between water and sediment. April 21, 1995.

9. PER WOIN, Xenobiotics in aquatic ecosystems: effects at different levels of organization. December 15, 1995.

10. GÖRAN EWALD, Role of lipids in the fate of organochlorine compounds in aquatic ecosystems. October 18, 1996.

11. JOHAN KNULST, Interfaces in aquatic ecosystems: Implications for transport and impact of anthropogenic compounds. December 13, 1996

12. GUDRUN BREMLE, Polychlorinated biphenyls (PCB) in a river ecosystem. April 25, 1997.

13. CHRISTER BERGWALL, Denitrification as an adaptive trait in soil and groundwater bacteria. November 14, 1997

14. ANNA WALLSTEDT, Temporal variation and phytotoxicity of Batatasin-III produced by Empetrum hermaphrodytum. November 27, 1998

15. DARIUS SABALIUNAS, Semipermeable membrane devices in monitoring of organic pollutants in the aquatic environment. April 28, 1999.

16. CECILIA AGRELL, Atmospheric transport of persistent organic pollutants to aquatic ecosystems. May 21, 1999

17. OLOF BERGLUND, The influence of ecological processes on the accumulation of persistent organochlorines in aquatic ecosystems. September 17, 1999

18. HELENA BJÖRN, Uptake, turn-over and distribution of chlorinated fatty acids in aquatic biota. October 1, 1999

19. RUEY-JANE FAN, Learning and memory in moths Plasticity in behaviour and neurophysiology. December 1, 2000.

20. FREDRIK ÖSTRAND, Behaviour of pine sawflies in relation to pheromone-based pest management. January 19, 2001.

21. MATTIAS LARSSON, Neural interfaces to the odour world of scarab beetles. March 2, 2001

22. CECILIA BACKE, Persistent organic pollutants in the atmosphere-spatial and temporal variations. May 4, 2001.

23. RICKARD IGNELL, Olfaction in desert locusts — Anatomy, function and plasticity of the central olfactory system. May 11, 2001.

24. CAMILLA RYNE, Pyralid moth reproduction: Communication, constraints & control. November 2, 2001.

25. DAINIUS PLEPYS, Odour-mediated nectar foraging in the silver Y moth, Autographa gamma. November 30, 2001.

26. DAVID ABRAHAM, Molecular aspects of pheromone evolution in moths. May 7, 2002.

27. GLENN SVENSSON, Disruption of moth mating behaviour by olfactory and acoustic cues. October 25, 2002.

28. LINA WENDT-RASCH, Ecological effects of pesticides in freshwater model ecosystems. February 28, 2003.

29. ARNOUT TER SCHURE, Polybrominated diphenylethers in the environment. October 3, 2003.

30. ELNA NILSSON, Movement patterns and displacement of a soil insect. May 28, 2004.

31. PER BENGTSON, Microbial mobilization and immobilization of soil nitrogen. June 4, 2004.

32. BETINA KOZLOWSKY SUZUKI, Effects of toxin-producing phytoplancton on copepods: feeding, reproduction and implications to the fate of toxins. June 7, 2004, Campus Helsingborg.

33. NIKLAS TÖRNEMAN, Spatial variability linking carbon resource heterogeneity and microorganisms; causes and consequences. May 13, 2005.

34. NIKLAS HOLMQVIST, Persistent organic pollutants in benthic foodwebs. June 10, 2005.

35. P.-O. CHRISTIAN OLSSON, Semiochemical-mediated attraction and oviposition in pyralid moths. November 11, 2005.

36. PARDIS PIRZADEH, Ecotoxicological assessment by microcosm tests on plankton communities. April 7, 2006.

37. CHRISTEL CARLSSON, Limitations and possibilities for microbial degradation of organic contaminants in aquifers. April 21, 2006.

38. MARIA PERSSON, The threat to the Baltic Salmon: a combination of persistent pollutants, parasites and oxidative stress. Nov 17, 2006.

39. GERMUND VON WOWERN, Circadian rhytms in moth sex pheromone communication. November 24, 2006.

40. LINA KRISTOFFERSEN, Getting to know Trioza apicalis (Homoptera: Psylloidea) – a specialist host-alternating insect with a tiny olfactory system December 1, 2006.

41. SÉVERINE JANSEN, Structure and function of odorant binding proteins and chemosensory proteins in moths. March 6, 2007.

42. MARIA STRANDH, Pheromones, genes & transcriptomes – a molecular analysis of moth sex pheromone production. November 30, 2007.

43. KATIA MONTENEGRO, Hierarchical responses to organic contaminants in aquatic ecotoxicological bioassays: from microcystins to biodegradation. September 23, 2008.